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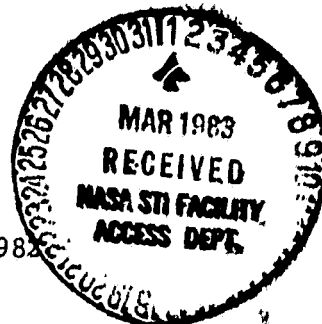
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(NASA-CR-169929) ORIGIN AND EVOLUTION OF
OSMOREGULATORY MECHANISMS IN BLUE-GREEN
ALGAE AS A FUNCTION OF METABOLIC AND
STRUCTURAL COMPLEXITY: REFLECTIONS OF
(University of Southern Illinois,

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Semiannual Status Report
of
Research Accomplished for the
Period June 15 - December 31, 1982
on the Project Entitled



'Origin and Evolution of Osmoregulatory
Mechanisms in Blue-Green Algae
as a Function of Metabolic and
Structural Complexity: Reflections of
Precambrian Paleobiology?'

Grant number: NAGW - 344

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Title: Origin and Evolution of Osmoregulatory Mechanisms in Blue-Green Algae (Cyanobacteria) as a Function of Metabolic and Structural Complexity: Reflections of Precambrian Paleobiology?

(NAGW - 344)

Objectives: The objectives of the study are:

- (1) To ascertain whether there exists any relationship between mode of nutrition in the cyanobacteria (i.e. photoautotrophic, photoheterotrophic or heterotrophic) expressed under aerobic or anaerobic conditions and the nature of the solutes employed for adjustment of intracellular water potential (osmoregulation);
- (2) To determine whether there exists any relationship between structural complexity in the cyanobacteria (unicellular, simple filamentous or multi-seriate branched) and the nature of the solutes employed for adjustment of intracellular water potential;
- (3) To conduct the studies on osmoregulation under environmental conditions that correspond to those of the major stages in the evolution of the Precambrian biosphere;
- (4) To identify and characterize the uptake and enzymatic mechanisms involved in the production and accumulation of the solutes employed in adjustment of intracellular water potential by representative nutritional and structural types of cyanobacteria, under the environmental conditions proposed in the previous objectives;
- (5) To determine whether differences in metabolism underlie the intracellular adjustment to lowered environmental water potential achieved by the addition of sodium chloride or non-ionic (penetrating or non-penetrating) solutes to the growth medium of cyanobacteria;
- (6) To ascertain whether genome size and other chemical characteristics of the genetic material influence the nature of the osmoregulatory response elicited under varying environmental conditions in the representative cyanobacteria;
- (7) To ascertain whether adjustment of intracellular water potential in cyanobacteria is a multiphasic process culminating in the accumulation of a solute (or solutes) compatible to enzymatic functioning; and to determine whether the degree of complexity in any multiphasic process is a function of metabolic, genetic or structural complexity;
- (8) To determine to what extent the inorganic and organic solutes accumulating during osmoregulation account for balancing the external water potential; and to ascertain whether the "sensing" of changes in environmental water potential is by a turgor-activated mechanism or not; and

- (9) To reconstruct a probable sequence of evolution of osmoregulatory mechanisms as a function of the adaptive interaction between the metabolic capabilities of Precambrian cyanobacteria and fluctuating water potential in ecosystems of a changing Precambrian biosphere.

Progress in achieving the stated objectives during the first year of present grant

Excellent progress has been made during the past seven months, since the start of the funded research. The following is a summary of this progress and the objectives partially achieved during the course of the research:

1. Twenty-four (24) of the twenty-nine cyanobacteria proposed for culture (in Table 2 of first year proposal) have been successfully cultured. A list of these is provided as Table 1.

It is beleived that the remaining five cyanobacteria (Synechococcus, ATCC# 29154; Synechococcus, ATCC# 29403; Fischerella, ATCC# 29161; Oscillatoria, PCC# 7805; LPP sp; ATCC# 29206) will be cultured in the remaining five months of this first year of the grant.

The above cultures are now in the actively growing condition, under a regular transfer protocol. This research required considerable experimentation to achieve the proper environmental conditions of light, temperature and physico-chemical characteristics of the culture media.

Our group worked closely with certain staff of the American Type Culture Collection (ATCC) facility. Personal communication with Dr. Stjepko Golubic of Boston University during the First Symposium on Chemical Evolution and the Origin of Life, organized by Dr. Donald L. DeVincenzi, NASA Headquarters also aided in solving some of the ancillary problems.

In addition, three additional blue-green algae ('cyanobacteria') provided by Dr. Lynn Margulis of Boston University during the same Symposium on Origin of Life at Ames Research Center, Moffett Field, California were also successfully cultured.

These cyanobacteria, in actively growing cultures, are now available for use by other scientists working within the same program funded by NASA. This represents, therefore, a valuable repository of the "organisms of the Precambrian" for immediate use by these scientists.

2. One third of the cyanobacteria (approximately) from the group (group E of Table 1) of facultative chemoheterotrophs are now in large scale culture for use in experimentation involving osmoregulatory solute determination and NMR studies.
3. The principal osmoregulatory solute (compatible solute as well) of the extremely halotolerant Aphanothece halophytica, has been identified as a betaine by NMR (Figure 1). This solute is found in

cellular concentrations of five to fifteen times those of all of the soluble carbohydrates and free amino acids combined (Table 2).

We have identified a nine-carbon polyol that also fluctuates with changes in external (medium) osmolarity. However, this solute accumulates in concentrations too low to serve a major osmoregulatory role.

The betaine content of A. halophytica not only increases dramatically with increasing NaCl molarity of the growth medium (Table 2) but also increases in response to rapid changes in medium osmolarity (i.e., upshock). Figure 2 depicts the change in this methylamine with upshock in the light and dark. Note that betaine content is higher in organisms grown or upshocked in media containing glycylglycine buffer than in media containing MES buffer. We have also found that betaine content falls with downshock, but not to initial levels. There is preliminary evidence that betaine is metabolized following downshock. Studies on this question are currently in progress.

4. Betaine synthesis in A. halophytica requires light (Figure 2). This organism is an obligate photoautotroph but does slowly deplasmolyze in the dark. However, growth will not continue in the dark.
5. Betaine is not merely a neutral osmoregulatory solute but also displays counteracting effects on salt-inhibition of enzyme activity. Glucose-6-phosphate dehydrogenase from A. halophytica was assayed in the presence of increasing concentrations of KCl (Figure 3). Approximately 50% inhibition of activity was obtained between 0.3 and 0.4M KCl. This concentration has been shown to occur in A. halophytica (see previous studies). Next, this enzyme was assayed in a medium containing 0.4M KCl plus either glycerol, proline or betaine (Figure 4). Although some relief from salt inhibition was obtained with proline and glycerol, betaine clearly was the superior counter-acting, compatible solute. Figure 5 depicts the range of protection afforded this enzyme by 2.0M betaine in assay media of increasing KCl-molarity. This range covers the range of KCl content that we previously found for this cyanobacterium.

Finally, the effect of the extent of methylation of glycine on protection against salt inhibition of glucose-6-Phosphate dehydrogenase was examined (Figure 6). There was found a clear relationship between degree of methylation and extent of salt counteracting effect.

6. The source (organism) of the enzyme is a factor in the extent of protection against salt inhibition afforded by betaine. Contrary to reports in the literature (82), a compatible, osmoregulatory solute will not protect a particular enzyme regardless of source. We obtained purified glucose-6-phosphate dehydrogenase from an eukaryotic organism, Torula yeast and another prokaryotic organism, Leuconostoc mesenteroides. The enzyme from the latter organism was several-fold more salt tolerant than the enzyme from the former organism. Betaine had dramatically different effects on the degree

of salt protection for the enzyme from different sources (Figure 7). The inherently more salt tolerant enzyme was actually inhibited by betaine.

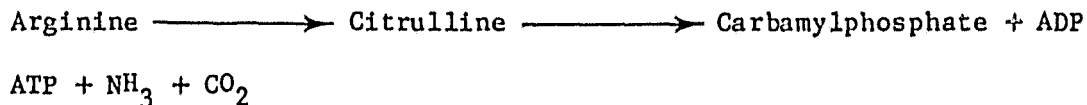
7. Betaine may exert its "counteracting effects" on salt inhibition not by "restoring activity" of salt inhibited enzymes, but by lowering the K_m of the enzyme per se. Figure 8 shows the effect of betaine on K_m , independent of the presence of high KCl in the assay medium. We had reported earlier that KCl inhibits enzyme activity in halotolerant blue-green algae by causing an increase in K_m . Betaine appears to "allow" enzyme function in the presence of high internal K^+ by greatly increasing the affinity of the enzyme for its substrate -- for enzymes from certain, but not all, organisms.
8. The changes, both quantitative and qualitative, in amino acids following upshock of the halotolerant, Aphanotheca halophytica, have been completely documented. The following major points can now be made concerning these changes:

a) The predominant free amino acids in the pool are glutamic> serine> glycine> aspartic> alanine; Table 3 and 4. Whereas the total free amino acids do not increase as the salinity of the growth medium increases (Table 2), an apparent increase occurs in this organism upon upshock in medium containing glycylglycine buffer (Table 3). No similar increase is observed in upshocked cyanobacteria in medium containing MES buffer. The increase under the former buffer condition appears in the "leucine fraction" of the analysis obtained by ion exchange within the Beckman 19CL Amino Acid Analyzer. However, we have now shown this "amino acid" to be the buffer glycylglycine by using High Voltage Paper Electrophoresis. It is obvious that the dipeptide glycylglycine itself is utilized by the alga during upshock. Note that the "leucine" does not increase in upshocked cells in MES buffer (Tables 3 and 4). There are reports of other bacteria utilizing the amino acids of their growth media under upshock conditions. Of even more interest to us is the consequence of glycylglycine accumulation during upshock. The amino acid proline accumulates in MES buffered-organisms following upshock. This is a major difference involving an amino acid known to be involved in the osmoregulatory mechanism (perhaps as a compatible solute) of eukaryotic and prokaryotic organisms. It should be noted also that glycylglycine is taken up from the upshock media under light, dark and dark anaerobic conditions (Table 5). Another major difference is that lysine greatly increases in cyanobacteria upshocked in glycylglycine, but not in cyanobacteria upshocked in MES buffered-medium.

b) The amino acids changing to the greatest extent with upshock are serine and glutamic acid. These are also the major amino acids of the free pool in this organism. The glutamic acid always decreases to a major extent and the serine concomitantly rises. The relationship is believed by the investigators to indicate a metabolic relationship between the two in which glutamic acid may serve as an amino donor for serine biosynthesis. Note also from Figures 9, 10 and 11, that this relationship exists in organisms upshocked in light, dark and dark-anaerobic conditions. In addition, it should be

noted that the relationship occurs independent of type of buffer present in the upshock media. We feel that these findings are of particular importance in view of the fact that serine is the precursor to betaine and that this amino acid is "triggered" to accumulate with increasing salinity under all environmental conditions tested. The significance of this observation to osmoregulation in cyanobacteria and to evolution of osmoregulatory mechanisms will be discussed in detail below.

c) Figure 11 shows another interesting feature of upshocking this cyanobacterium under anaerobic conditions. As previously mentioned, one reported (17) possible source of energy in cyanobacteria under dark anaerobic conditions is the following pathway:



The reserve polymer of aspartic acid and arginine called cyanophycin could break down under anaerobic conditions (dark) to furnish arginine. As shown in figure 11, arginine does appear in relatively large quantities under this condition and NH_3 (ammonia), the breakdown product of its use, also accumulates. The cyanobacteria do, then, have a primitive source of energy (ATP) derived from a pathway capable of functioning in the absence of oxygen and light.

Our results indicate that at least two possible osmoregulatory solutes are synthesized in this cyanobacterium, betaine and proline. Proline will accumulate (in media without glycylglycine) in both light and dark, betaine will not (Figure 2).

9. The changes in total soluble carbohydrates, reducing carbohydrates, total amino acids and individual amino acids following upshock in light and dark of three different structural types of cyanobacteria have been determined. These were the unicellular Synechocystis the filamentous, LPP; and the branched Fischerella. The major findings of this experimentation are as follows:

a) No betaine accumulated following upshock in NaCl (sufficient for plasmolysis and recovery) in any of the three structural types of cyanobacteria. These results and several months of preliminary experimentation to "find" betaines in other cyanobacteria, have led us to tentatively conclude that betaine is a compatible, osmoregulatory solute formed in halotolerant and halophilic oxygenic photoautotrophs, but not in fresh water organisms of the same type. All three of the structural types so tested were of fresh water origin.

b) Quantitatively speaking, the solute class increasing to the greatest extent in the Synechococcus, LPP (Oscillatoria type) and Fischerella was that of the non-reducing carbohydrates. Non-reducing carbohydrate amount is obtained by subtracting reducing carbohydrates from total soluble carbohydrates in our analysis protocol.

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Figures 12-17 show the changes in total and reducing carbohydrates in both light and dark for the three cyanobacteria, seven and 24-hours after upshock.

Clearly, the evolutionarily most advanced Fischerella, contains the greatest amount of non-reducing carbohydrate (Figures 12 and 13). In addition, the non-reducing carbohydrate fraction (we believe one molecular type) more than doubles with upshock after only seven hours. Furthermore, this increase occurs in both light and dark. The carbohydrate(s) synthesis then proceeds without an immediate light requirement. There is little or no increase in reducing sugars following upshock in the Fischerella.

A much different effect of upshock is seen in the less evolutionarily advanced LPP (Figures 14 and 15). In this case, non-reducing carbohydrates (again, we believe one chemical species) more than doubles after six hours of upshock, but, only in the light. Deplasmolysis is also very much slower in the dark. Again, there is no appreciable increase in reducing sugars with upshock.

Finally, the unicellular cyanobacterium Synechocystis behaves much the same way as the filamentous LPP with respect to total carbohydrate. However, under light conditions only, the reducing carbohydrates (but not non-reducing fraction) increase with upshock.

It should be noted that only the Fischerella is capable of growing in the dark on a carbohydrate source (i.e. chemoheterotrophically).

Studies are currently in progress to identify the individual carbohydrates in the various fractions.

c) The changes in free amino acids in the three fresh-water cyanobacteria did not correlate with those found for the carbohydrates. These changes are quantitatively represented in Figures 18-20. The greatest changes occur in the unicellular Synechocystis and filamentous LPP. No increase in total amino acids were observed for the Fischerella.

Figure 18 shows the approximate 50% increase in total amino acids in Synechocystis after seven hours of upshock. This occurred both in light and dark. The levels returned to near control after 24 hours.

Figure 19 shows the much greater increases in total amino acids following upshock of the filamentous LPP. In this case, as in the previous one, the changes are very much greater in the light than in the dark. In addition, the levels remain high, and even double the non-upshocked control, but only in the light.

Figure 20 shows a slight, transitory change in amino acids in the upshocked Fischerella in the light, but a return to control levels after 24 hours. Clearly, amino acids are not as quantitatively important in this rather advanced cyanobacterium.

d) Qualitative changes in the amino acids of the three fresh water cyanobacteria proved to be more interesting. Tables 6, 7 and 8 show that for all three organisms the five major (quantitatively amino acids are glutamic acid, aspartic acid, serine, alanine and proline. Glutamic acid is always most abundant, followed by the other four in orders varying among the three species. Four of the same amino acids are the most prevalent in Aphanothece as well.

Figures 21, 22 and 23 depict the changes following upshock in those amino acids most affected by the treatment.

First, some generalizations are evident. In all organisms, proline, arginine and lysine increase in the light during upshock. However, proline and arginine require light for their synthesis, except in the case of Fischerella.

In both Synechocystis and Fischerella glutamic acid decreases following upshock, but increases in the filamentous LPP, especially in the dark.

Quantitatively, proline is the major amino acid changing with changing salinity in both Synechocystis and the LPP, but only in the light. However, proline greatly increases only in the dark in Fischerella. Obviously, the large increase in amino acids in the LPP Alga following upshock, is due almost entirely to proline and glycine (Figure 22).

Significance of the Research Accomplished During the Past Seven Months

The specific findings reported above are significant with respect to qualification of certain conclusions concerning cyanobacteria and the nature of compatible solutes that have been recently reported in the literature.

First, there exists the problem of too closely grouping the cyanobacteria (or blue-green algae) to other gram-negative bacteria, as is frequently done (1). Gram-negative bacteria (not cyanobacteria however) show increases in glutamic acid and/or proline and gamma aminobutyric acid (GABA) following upshock. Obviously from our data this does not occur. In fact, the halo-tolerant cyanobacterium Aphanothece, always showed a decrease in glutamate with upshock concomitant to an increase in serine, even as K^+ was increasing. Furthermore, we have been unable to find any GABA, even at the nanomole level in this organism.

Secondly, our data show that proline and glycine, two solutes often found in osmoregulating eukaryotes, do accumulate in certain cyanobacteria under certain environmental conditions. In one case (Aphanothece) both betaine and proline accumulate.

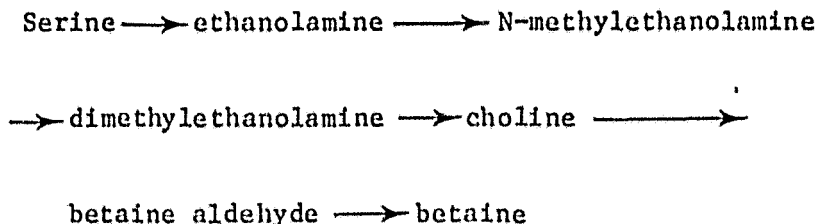
As previously reported, we appear to also find, again in certain cyanobacteria, the glucosylglycerol involved in osmoregulation. However, a recent review in Science by Yancey, et al. (3) reports only this osmoregulatory solute (osmolyte) for the cyanobacteria. The major thesis of this particular article was the great uniformity (and lack of diversity) of

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chemicals evolved for the purpose of osmoregulation. We believe, from our initial data, that there is considerable diversity in osmoregulatory solutes employed by the cyanobacteria from adaptive evolution to water and saline stress.

Thirdly, and of considerable importance to non-blue-green bacteria, amino acids in the growth (culture) medium may be taken up (or diffuse in) during upshock. These amino acids may, if the case involving Aphanothece is repeated, influence the nature of the solutes formed during osmoregulation. Early heterotrophic bacteria, which were probably the earth's first prokaryotes, almost certainly had amino acids available to them. One of the most common of these was almost certainly glycine, and perhaps, the dipeptide, glycylglycine. These amino acids may have directed the pathways of osmoregulatory solute production.

Fourth, recent research on the pathway of betaine synthesis in higher plants by Andrew Hanson and his coworkers at Michigan State (2) confirms that serine is the precursor betaine. The pathway is as follows:



However, these investigators have focused their attention to the steps in this pathway that are responsive to increasing water stress or salinity--after the formation of serine. They further note that betaine synthesis requires light, especially for the formation of one carbon metabolite derivatives of formic acid. Our data certainly agree with those indicating a light requirement for betaine synthesis. However, we believe that our data also show that the decreased water potential and/or salinity trigger the synthesis of serine. Serine formation is the limiting step for betaine synthesis and later stages in the pathway may or may not be stimulated by decreasing water potential or increasing internal salinity. Furthermore, we believe that the serine, while certainly not of photorespiratory origin, probably comes from the non-phosphorylated, D-glyceric pathway.

Fifth, and finally, our data also challenge, or at least qualify, the statement made by Yancey et al (3, p. 1217) that the "counteracting effects [to salt inhibition of enzymes] [of betaine] are independent of the species source of protein. Mammals, teleost, amphibian and elasmobranch proteins respond similarly in the presence of counteracting solutes, regardless of whether they experience these solutes in vivo" and on p. 1221.

"Through the use of compatible solute systems, proteins are able to work in the presence of high or variable solute concentrations, and the [genetic] modifications of vast numbers of proteins is avoided."

We agree with the importance of their assessment of the evolutionary role of compatible solutes, as this gives even more justification for the objectives of our proposed research. However, our experience (reported here)

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with glycine-betaine and glucose-6-phosphate dehydrogenase from different sources, leads us to call for more experimentation on protein modification in organisms employing greatly different osmoregulatory systems.

Presentations and Publications Resulting from the Research.

- (1) Two abstracts will be published in the journal Plant Physiology of papers presented at the Annual Meeting of the American Society of Plant Physiologists August 10, 1983 at

These abstracts are entitled:

- (a) Effect of betaine on the activity of salt-inhibited glucose-6-phosphate dehydrogenase from the halotolerant cyanobacterium, Aphanothece halophytica and two other non-halophilic microorganisms - by Ken Pavlicek and John H. Yopp
- (b) Influence of the environment on the amino acids and betaine synthesized by the halotolerant cyanobacterium, Aphanothece halophytica during water stress - by John H. Yopp, Ken Pavlicek and Rebecca Pavlides.

(2) Manuscripts in preparation

- (a) Yopp, J. H. Role of betaine and amino acids in osmoregulation by the extremely halotolerant cyanobacterium, Aphanothece halophytica - to be submitted to Origins of Life - 1983
- (b) Tomlinson, J. and J. H. Yopp. Solute changes during osmoregulation in cyanobacteria of different structural and ecological types - to be submitted to Journal of Phycology - 1983

(3) Published abstracts from NASA-supported research:

- (a) Pavlicek, K. V. and J. H. Yopp 1982. Betaine as a compatible solute in the complete relief of salt inhibition of glucose-6-phosphate dehydrogenase from a halophilic blue-green alga. Plant Physiol. 69(4): 56.

Table 1

Nutritional and Morphological Types of Blue-green Algae (Cyanobacteria) Successfully
Cultured in Defined Nutrient Medium from June 15-December 30, 1982

Nutritional type and strain genus	Morphological type, mean genome size, and mol% GC ¹	Taxonomic Group	Culture collection(s) ³ and collection number
A. Obligate photoautotrophs			
<u>Synechococcus</u>	unicellular, 1.57 x 10 ⁹ daltons; 47-56 mol% GC	Chroococcaceae; Section I	PCC #6311; ATCC # 27145
<u>Synechococcus</u>	unicellular, 2.4 x 10 ⁹ daltons; 66-71 mol% GC	Chroococcaceae; Section I	PCC # 7009; ATCC #29203
<u>Aphanothece halophytica</u>	unicellular	Chroococcaceae; Section I	Botany Dept.; Southern Ill. Univ., isolate YT-2.
<u>Oscillatoria</u>	filamentous; 4.38 x 10 ⁹ daltons; 40-50 mol% GC	Oscillatoriaceae; Section III	PCC # 6304; ATCC # 27930
LPP Group	filamentous; 2.63 x 10 ⁹ daltons; 42-52 mol% GC	Oscillatoriaceae; Section III	PCC # 6703; ATCC # 27907
LPP Group	filamentous, 5.19 x 10 ⁹ daltons; 42-52 mol% GC	Oscillatoriaceae; Section III	PCC # 7408; ATCC # 29344

B. Obligate photoautotrophs-
nitrogen fixing

Table 1 (cont'd)

D. Facultative photoheterotrophs-
nitrogen fixing

LPP Group	filamentous; 3.7 x 10 ⁹ daltons; 42-52 mol% GC	Oscillatoriaceae; PCC # 6409; ATCC #29119 Section III
<u>Calothrix</u>	filamentous; hetero- cystous; 5.23 x 10 ⁹ daltons; 40-44 mol% GC	PCC # 7103, ATCC # 27905 Nostocaceae; Section IV
<u>Calothrix</u>	filamentous; hetero- cystous; 8.58 x 10 ⁹ daltons; 40-44 mol% GC	PCC #7102; ATCC # 27901 Nostocaceae; Section IV
<u>Fischerella</u>	branched-filamentous, Stigonemataceae; heterocystous; 3.62 x Section V 10 ⁹ daltons; 42-46 mol% GC	PCC # 73103, ATCC # 29114 UTEX 1301

E. Facultative chemoheterotrophs

<u>Synechocystis</u>	unicellular; 2.34 x 10 ⁹ daltons; 42-48 mol% GC	Chroococcaceae; PCC # 6714; ATCC # 27178 Section I
<u>Synechocystis</u>	unicellular; 1.79 x 10 ⁹ daltons; 42-48 mol% GC	Chroococcaceae; PCC # 6803; ATCC # 27184 Section I
Oscillatoria	filamentous, 3.86 x 10 ⁹ daltons; 40-50 mol% GC (nitrogen fixing)	Oscillatoriaceae; PCC # 6412; ATCC # 29205 Section III UTEX 1546

Table 1 (cont'd)

<u>Gloeotheca</u>	unicellular; 5.02 x 10 ⁹ daltons; 40-43 mol% GC	Chroococcaceae; Section I	PCC # 6501; ATCC # 27151, UTEX 1938
LPP Group	filamentous; 3.77 x 10 ⁹ daltons; 44-52 mol% GC	Oscillatoriaceae; Section III	PCC # 7408; ATCC # 29344
<u>Anabaena</u>	filamentous heterog- cystous; 2.17 x 10 ⁹ daltons; 38-44 mol% GC	Nostocaceae Section IV	PCC # 7122; ATCC # 27899 UTEX TM B 629
<u>Nostoc</u>	filamentous, heterog- cystous; 4.00 x 10 ⁹ daltons; 39-45 mol% GC	Nostocaceae Section IV	PCC # 6719; ATCC # 29105
<u>Nostoc</u>	filamentous, heterog- cystous; 6.42 x 10 ⁹ daltons; 39-45 mol% GC	Nostocaceae Section IV	PCC # 7422; ATCC # 29312

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C. Facultative photoheterotrophs

<u>Synechocystis</u>	unicellular; 1.79 x 10 ⁹ daltons; 35-37 mol% GC	Chroococcaceae; Section I	PCC # 6803; ATCC # 27184
<u>Synechocystis</u>	unicellular; 3.50 x 10 ⁹ daltons; 42-48 mol% GC	Chroococcaceae; Section I	PCC # 7509; ATCC # 29235
LPP Group	filamentous; 2.58 x 10 ⁹ daltons; 53-59 mol% GC	Oscillatoriaceae Section III	PCC # 7407; ATCC # 29126

Table 1 (cont'd)

<u>Calothrix</u>	filamentous, hetero- cystous; 5.23 x 109 daltons; 40-44 mol% GC.	Nostocaceae; Section IV	PCC # 7103; ATCC # 27905
<u>Calothrix</u>	filamentous, hetero- cystous; 7.75 x 109 daltons; 40-44 mol% GC	Nostocaceae Section IV	PCC # 7101, ATCC # 27914
<u>Clorogloeopsis fritschii</u>	filamentous, division in more than one plane 5.24 x 109 daltons; 42-43 mol% GC (heterocystous)	Stigonemataceae; Section V	PCC # 6718; ATCC # 27181

- 1 Genome size and mol% GC is given in Rippka et al and Herdman, et al .
- 2 Taxonomic group is according to classical position (Family) and Section number given by Rippka et al which was determined by developmental DNA and physiological characteristics.
- 3 Pasteur Culture Collection (PCC) and American Type Culture Collection (ATCC) numbers according to Rippka et al .
- 4 Placed by Rippka et al in Section I, Chroococcaceae.

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Table 2

Major Solute Content of A. halophytica Grown in Media of Increasing NaCl Salinity

NaCl Molarity of Medium	Solute Class*			
	total free amino acids mg x 10 ⁻⁷ per cell†	total soluble carbohydrates mg x 10 ⁻⁷ per cell††	total reducing carbohydrates mg x 10 ⁻⁷ per cell	glycine-betaine mg x 10 ⁻⁷ per cell
Glycyl-glycine buffered				
1	2.83	2.83	0.51	28.0
2	3.31	3.01	0.53	44.0
3	4.36	3.37	0.59	123.0
4	7.53	**	—	130.0
MES buffered				
1	2.56	—	—	36.2
3	1.23	—	—	67.6

* all values are averages of triplicate determinations

** not determined

† values are leucine equivalents

†† values of all carbohydrates are glucose equivalents

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Table 3

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Changes in Free Amino Acids* in A. halophytica Grown in Either Glycylglycine or MES Buffer Following Upshock (1.0m NaCl 2.75m NaCl - Medium) in the Light

Amino Acid	Buffer Type					
	Gly-Gly			MES		
	Initial	24 hr	72 hr	Initial	24 hr	72 hr
Glutamic Acid	21.00	8.09	9.86	17.30	6.25	6.58
Serine	4.12	8.14	12.70	4.92	8.11	6.04
Glycine	1.42	3.59	2.22	1.66	1.93	0.75
Aspartic Acid	1.41	1.41	1.66	1.55	1.32	0.97
Alanine Acid	0.86	0.11	0.86	1.63	1.32	0.64
Lysine	0.47	2.12	0.55	0.73	0.74	0.39
Valine	0.44	0.10	0.51	0.21	0.56	0.29
Leucine†	0.41	7.63	11.80	0.49	0.49	0.23
Histidine	0.34	0.36	0.35	0.42	0.42	_____
Threonine	0.30	_____	_____	_____	_____	_____
Cysteine	0.26	0.40	0.22	_____	0.21	_____
Isoleucine	0.25	0.13	0.26	0.34	0.33	_____
Phenylalanine	0.13	0.14	0.18	0.14	0.14	0.11
Ammonia	0.09	0.72	0.74	0.54	0.58	0.49
Tyrosine	_____	0.09	0.19	_____	_____	0.12
Methionine	_____	_____	_____	0.19	_____	0.18
Arginine	_____	_____	_____	_____	_____	_____
Proline	_____	_____	_____	_____	1.8	2.16

* Concentrations are expressed as mg x 10⁻⁸ per cell

† This amino acid co-chromatographs with the buffer glycylglycine; the increase observed in this buffer for leucine is then, actually glycylglycine.

Table 4

ORIGINAL PAGE IS
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or MES Buffer Following Upshock (1.0m NaCl 2.75m NaCl - Medium) in the Dark

Amino Acid	Buffer Type					
	Initial	Gly-Gly 24 hr	72 hr	Initial	MES 24 hr	72 hr
Glutamic Acid	21.00	6.33	11.90	17.30	16.40	11.50
Serine	4.12	13.50	13.20	4.92	6.82	10.50
Glycine	1.42	1.52	2.30	1.66	1.18	0.46
Aspartic Acid	1.41	1.48	1.47	1.55	1.16	1.27
Alanine	0.86	0.74	0.65	1.63	0.99	0.82
Lysine	0.47	1.16	1.86	0.73	1.06	0.99
Valine	0.44	0.54	0.48	0.21	0.47	0.29
Leucine†	0.41	7.29	9.66	0.49	0.45	0.21
Histidine	0.34	0.31	_____	0.42	0.29	_____
Threonine	0.30	_____	_____	_____	_____	_____
Cysteine	0.26	_____	_____	_____	_____	_____
Isoleucine	0.25	0.23	0.19	0.34	0.24	_____
Phenylalanine	0.13	0.09	0.07	0.14	0.18	_____
Ammonia	0.09	0.81	0.71	0.54	0.63	0.67
Tyrosine	_____	_____	_____	_____	0.47	_____
Methionine	_____	_____	_____	0.19	_____	_____
Arginine	_____	_____	0.49	_____	_____	_____
Proline	_____	_____	_____	_____	1.94	2.62

* Concentrations are expressed as $\text{mg} \times 10^{-8}$ per cell

† This amino acid co-chromatographs with the buffer glycylglycine; the increase observed in this buffer for leucine is, then, actually glycylglycine.

Table 5

Changes in Free Amino Acids in A. halophytica As a Function of Light and Oxygen Availability
Following Upshock (1.0m NaCl 2.75m NaCl-Medium)

Amino Acid	Light Aerobic			Treatment Regime			Dark Anaerobic		
	Initial	24 hr	72 hr	Initial	24 hr	72 hr	Initial	24 hr	72 hr
Glutamic Acid	21.00	8.09	9.86	21.00	6.33	11.90	22.40	28.30	1.30
Serine	4.12	8.14	12.70	4.12	13.50	13.20	2.43	5.71	11.30
Glycine	1.42	3.59	2.22	1.42	1.52	2.30	0.43	0.51	0.41
Aspartic Acid	1.41	1.41	1.66	1.41	1.48	1.47	0.83	0.76	0.90
Alanine	0.86	0.11	0.86	0.86	0.74	0.65	0.25	0.64	0.14
Lysine	0.47	2.12	0.55	0.47	1.16	1.86	0.68	0.40	0.50
Valine	0.44	0.10	0.51	0.44	0.54	0.48	0.15	0.22	0.15
Leucine†	0.41	7.63	11.80	0.41	7.29	9.66	0.87	12.80	19.60
Histidine	0.34	0.36	0.35	0.34	0.31	_____	_____	0.11	0.06
Threonine	0.30	_____	_____	0.30	_____	_____	0.07	0.08	_____
Cysteine	0.26	0.40	0.22	0.26	_____	_____	_____	_____	_____
Isoleucine	0.25	0.13	0.26	0.25	0.23	0.19	_____	_____	_____
Phenylalanine	0.13	0.14	0.18	0.13	0.09	0.07	_____	_____	_____
Ammonia	0.09	0.72	0.74	0.09	0.81	0.71	0.34	0.43	1.05
Tyrosine	_____	0.09	0.19	_____	_____	_____	_____	0.28	0.26
Methionine	_____	_____	_____	_____	_____	_____	_____	_____	_____
Arginine	_____	_____	_____	_____	_____	0.49	_____	0.19	0.43
Proline	_____	_____	_____	_____	_____	_____	_____	_____	_____

* Concentrations are expressed as mg x 10⁻⁸ per cell

† This amino acid co-chromatographs with the buffer glycylglycine, the increase observed in this buffer for leucine is, then, actually glycylglycine

Table 6

Changes in Free Amino Acids* in Synechouptis sp. As a Function of Light Following Upshock

Amino Acid	Treatment Regime and Time					
	Initial	Light 6 hr	24 hr	Initial	Dark 6 hr	24 hr
Glutamic Acid	1.18	1.25	0.91	1.12	1.15	1.29
Serine	0.34	0.35	0.18	0.14	0.16	0.16
Aspartic Acid	0.23	0.35	0.19	0.09	tr	0.13
Alanine	0.18	0.16	0.16	0.06	0.12	0.06
Proline	0.11	0.38	0.67	tr	tr	0.06
Valine	0.09	0.07	0.07	0.06	0.06	0.05
Threonine	0.08	0.12	0.12	0.05	0.06	0.07
Lysine	0.07	0.16	0.13	0.04	0.08	0.12
Glycine	0.07	0.19	0.13	0.06	0.10	0.09
Histidine	0.07	0.07	0.05	0.05	0.05	0.06
Leucine	0.03	0.03	0.03	0.02	0.03	tr
Tyrosine	0.02	0.08	tr	tr	tr	tr
Phenylalanine	0.02	0.02	tr	0.02	tr	tr
Isoleucine	0.02	0.02	0.01	tr	0.01	0.01
Arginine	0.02	0.04	0.06	tr	0.01	tr

* Concentrations are given in mg per g dry weight algae

Table 7

ORIGINAL PAGE IS
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Upshock

Amino Acid	Treatment Regime and Time					
	Initial	Light 6 hr	24 hr	Initial	Dark 6 hr	24 hr
Glutamic Acid	0.48	1.15	0.56	0.48	0.84	0.75
Serine	0.04	0.11	0.11	0.06	0.07	0.04
Aspartic Acid	0.16	0.19	0.08	0.10	0.12	0.12
Alanine	0.05	0.08	0.06	0.06	0.10	0.06
Proline	0.04	0.03	0.35	0.02	tr	tr
Valine	0.02	0.03	0.02	0.02	0.03	0.02
Threonine	0.02	0.07	0.07	0.02	0.03	0.02
Lysine	0.01	0.05	0.03	0.02	0.04	0.04
Glycine	0.03	0.43	0.25	0.03	0.04	0.03
Histidine	tr	0.01	0.01	0.01	0.01	0.01
Leucine	0.01	0.02	0.01	0.01	0.01	0.01
Tyrosine	0.01	0.03	0.06	0.02	0.01	0.01
Phenylalanine	tr	0.01	0.01	0.01	tr	tr
Isoleucine	0.01	0.01	0.01	0.01	0.01	tr
Arginine	0.02	0.07	0.03	tr	0.02	0.01

* Concentrations are given in mg per g dry weight algae

Table 8

Changes in Free Amino Acids in Fischerella muscicola As a Function of Light Following Upshock

Amino Acid	Treatment Regime and Time					
	Initial	Light 6 hr	24 hr	Initial	Dark 6 hr	24 hr
Glutamic Acid	3.11	2.16	1.68	2.16	1.64	2.52
Serine	0.45	0.55	0.29	0.56	0.55	0.63
Aspartic Acid	0.45	0.65	0.28	0.37	0.70	0.29
Alanine	0.67	0.62	0.34	0.86	0.46	0.47
Proline	1.62	1.91	1.67	0.83	1.19	0.65
Valine	0.08	0.07	0.06	0.28	0.22	0.12
Threonine	0.12	0.16	0.07	0.14	0.13	0.14
Lysine	0.20	0.42	0.38	0.22	0.36	0.19
Glycine	0.32	0.21	0.13	0.44	0.27	0.43
Histidine	0.02	0.04	0.02	0.03	0.02	0.02
Leucine	0.05	0.05	0.03	0.11	0.07	0.17
Tyrosine	0.09	0.17	0.12	0.10	0.04	0.08
Phenylalanine	0.04	0.04	0.02	0.06	0.04	0.05
Isoleucine	0.03	0.03	0.03	0.06	0.03	0.04
Arginine	0.13	0.16	0.30	0.16	0.13	0.16

* Concentrations are given in mg per g dry weight algae

Figure 1. Carbon-13 Nuclear Magnetic Resonance Spectrum of Glycine Betaine
Using 0.5 ml Microcell.

NIC

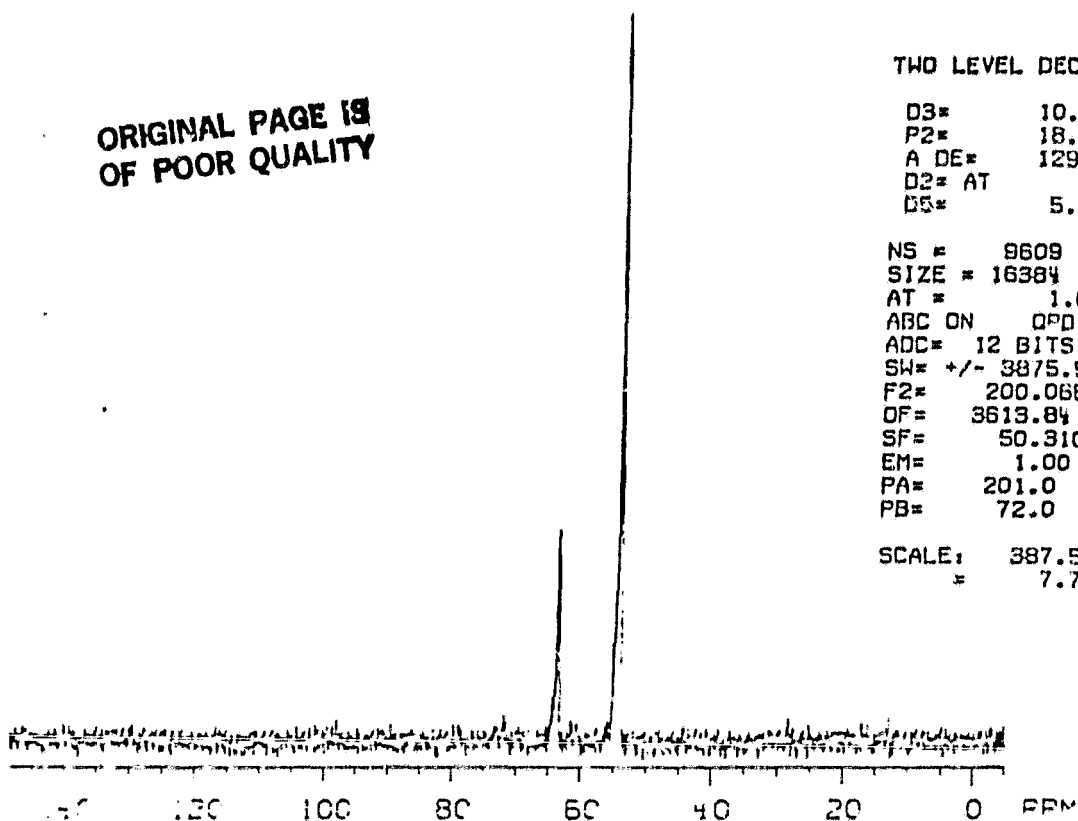
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TWO LEVEL DECOUPLING

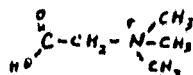
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OF= 3613.84
SF= 50.310349
EM= 1.00
PA= 201.0
PB= 72.0

SCALE: 387.59 HZ/CM
= 7.7041 PPM/CM



STANDARD
"0.5 cc cell" sample



CH₃NH marker

NIC

TWO LEVEL DECOUPLING

D3= 10.00 MSEC
P2= 18.00 USEC
A DE= 129 USEC
D2= AT
D5= 10.00 SEC

NS = 5658
SIZE = 16384
AT = 1.06 SEC
ABC ON QPD ON = 1
ADC= 12 BITS AI= 6
SW= +/- 3875.96 DW= 129
F2= 200.066240
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PB= 94.2

SCALE: 387.59 HZ/CM
= 7.7041 PPM/CM

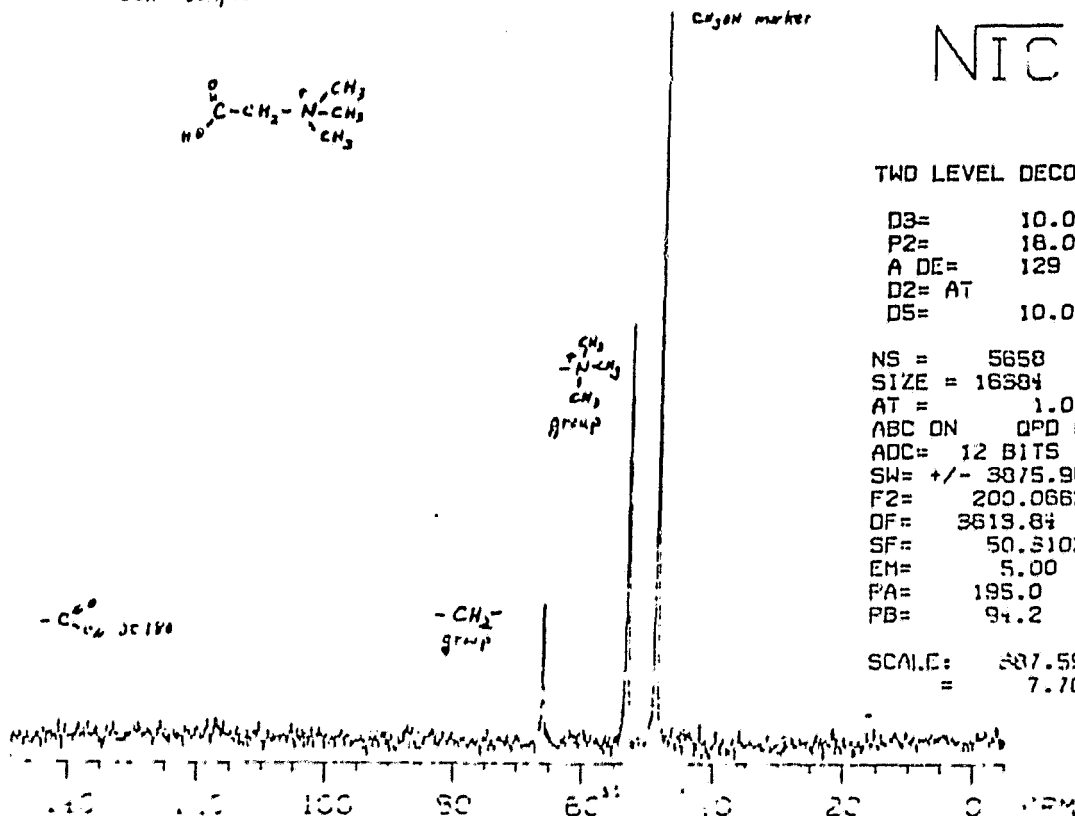


Figure 2. Changes in Betaine Content of Aphanothece halophytica After Upshock in Light and Dark Conditions and in Either Glycylglycine or MES Buffer.

CHANGES IN GLYCINE-BETAINE LEVELS IN *A. halophytica*
 FOLLOWING UPSHOCK (1.0M NaCl → 2.75M NaCl- MEDIUM)
 IN LIGHT AND DARK IN BOTH GLYCYL - GLYCINE AND MES BUFFERS

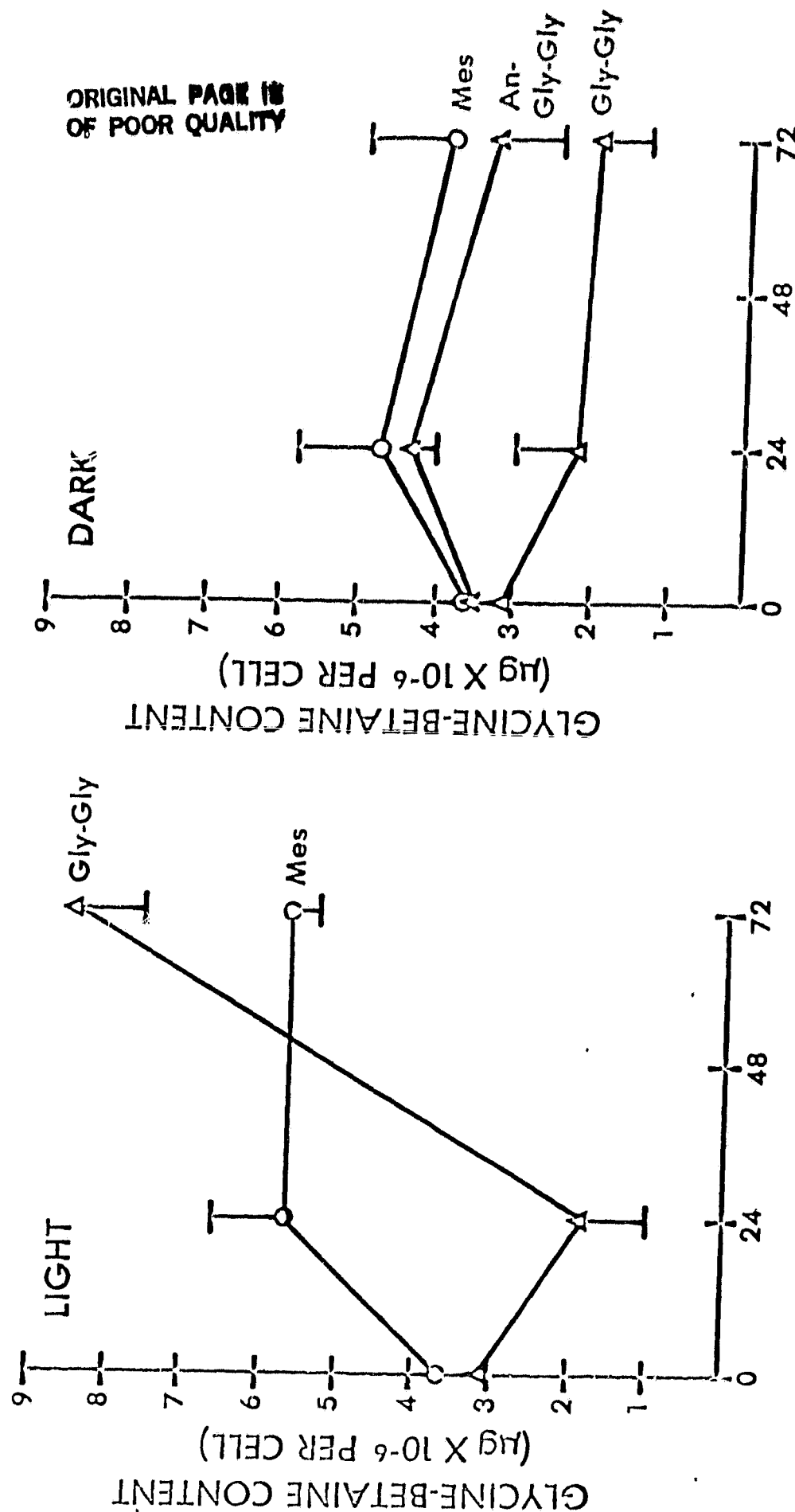
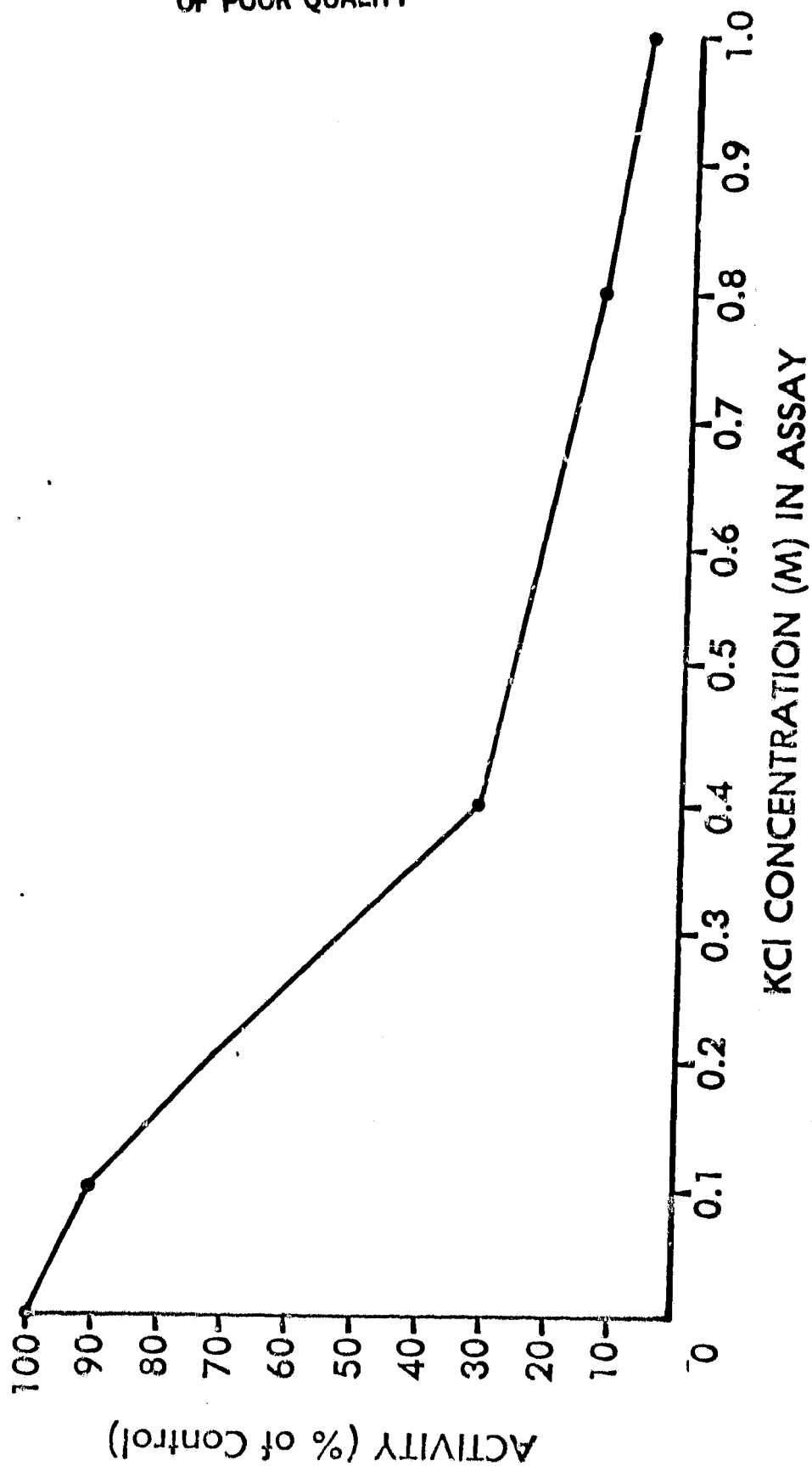


Figure 3. Effect of Increasing Concentrations of KCl on in vitro Activity of Glucose-6-Phosphate Dehydrogenase from Aphanothece halophytica

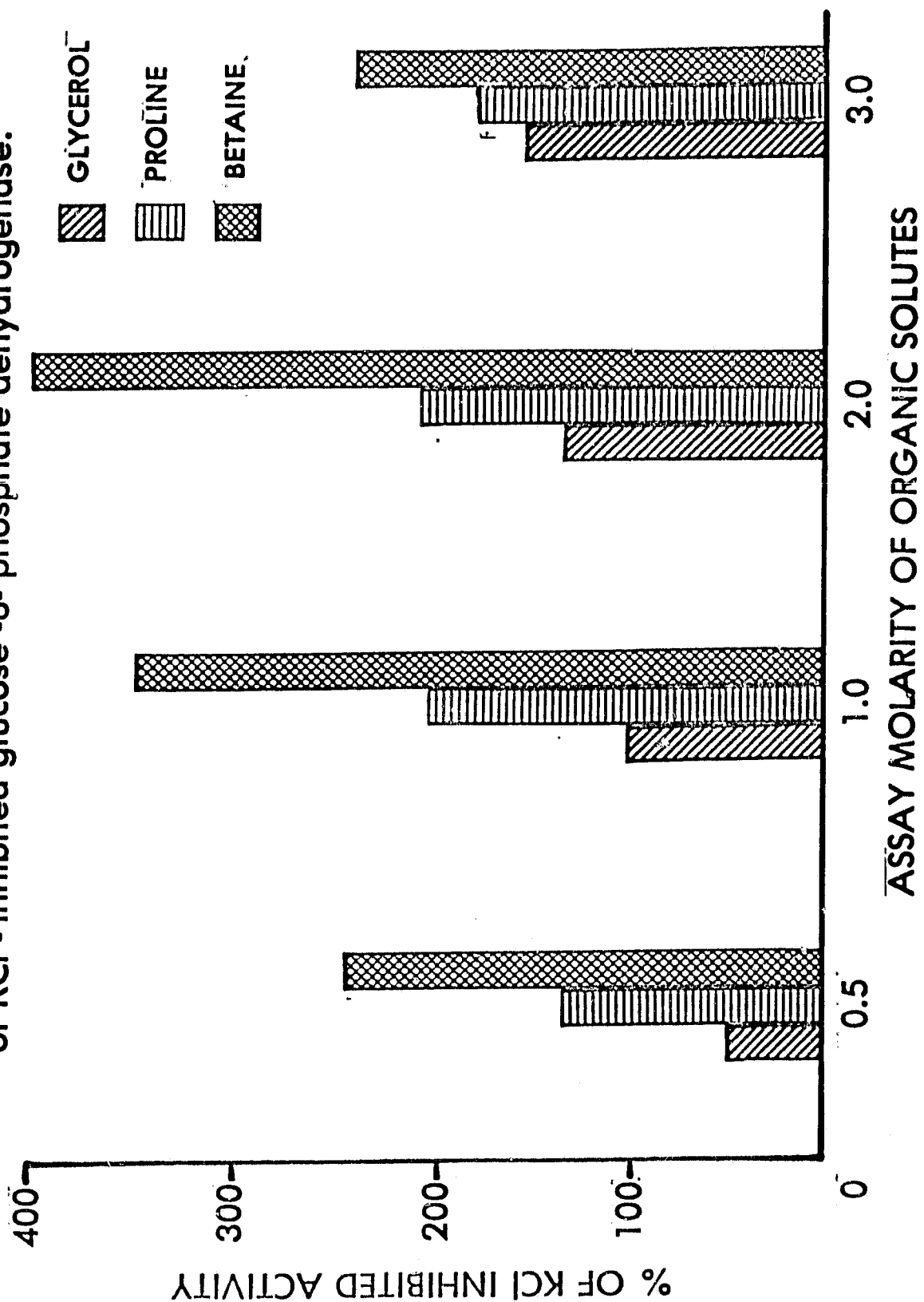
Effects of increasing concentrations of KCl in the assay medium
on the activity of glucose -6- phosphate dehydrogenase.



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Figure 4. Effects of Increasing Concentrations of Various Osmotica on the Activity of KCl - Inhibited Glucose-6-Phosphate Dehydrogenase from Aphanothece halophytica

Effects of increasing concentrations of various organic osmotica on the activity of KCl - inhibited glucose -6- phosphate dehydrogenase.



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Figure 5. Effects of Glycine Betaine (2.0M) on the Activity of Glucose-6-Phosphate Dehydrogenase Activity of Aphanothece halophytica in Increasing KCl Content of the Assay Medium

Effects of 2 M betaine on the activity of glucose -6- phosphate
dehydrogenase in increasing KCl content of the assay medium.

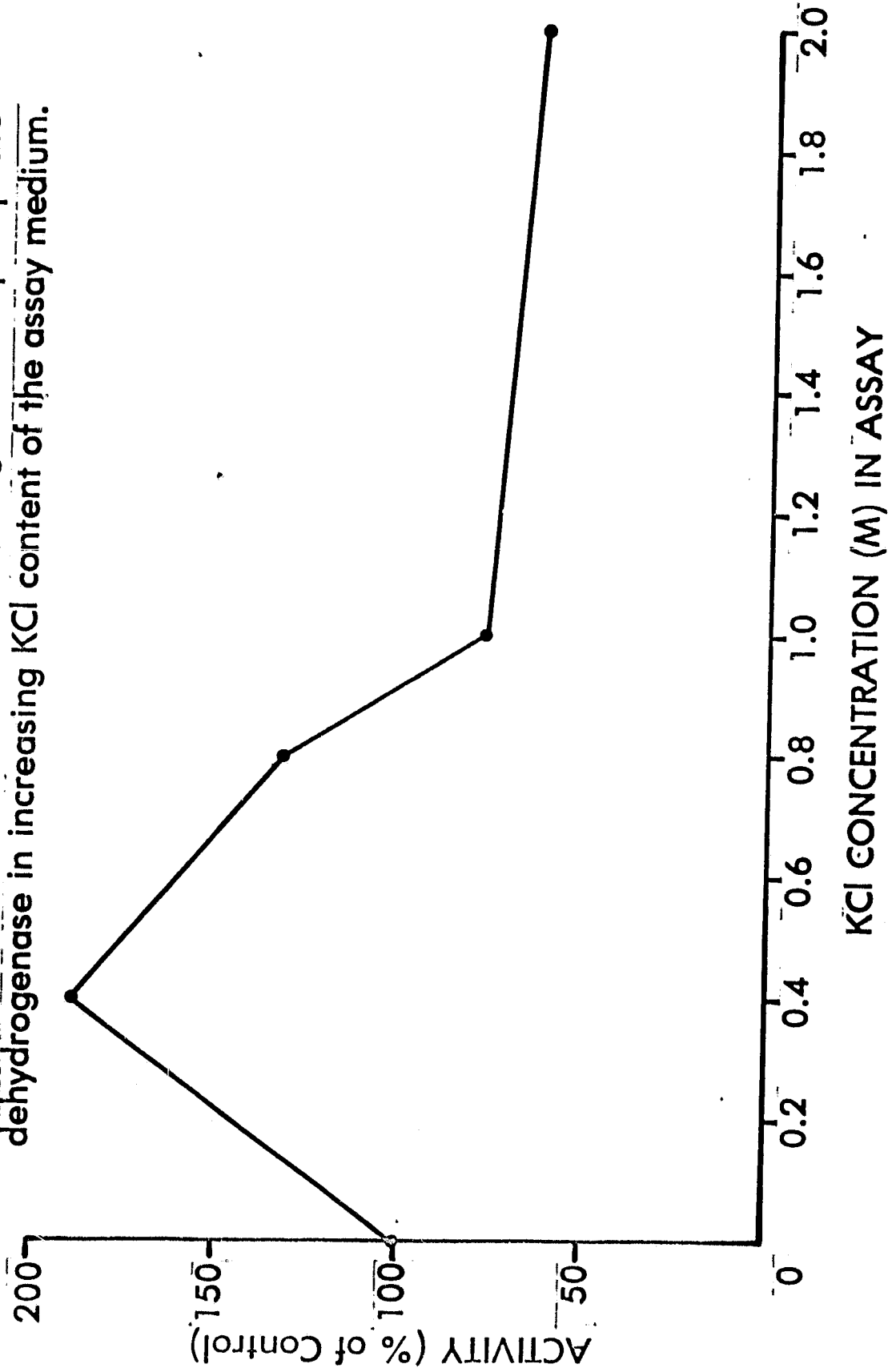


Figure 6. Effects of Successively Methylated Derivatives of Glycine (1.0M) on the Activity of KCl - Inhibited Glucose-6-Phosphate Dehydrogenase from Aphanothece halophytica

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Effects of 1 M assay concentrations of successively methylated derivatives of glycine on activity of KCl-inhibited glucose -6-phosphate dehydrogenase.

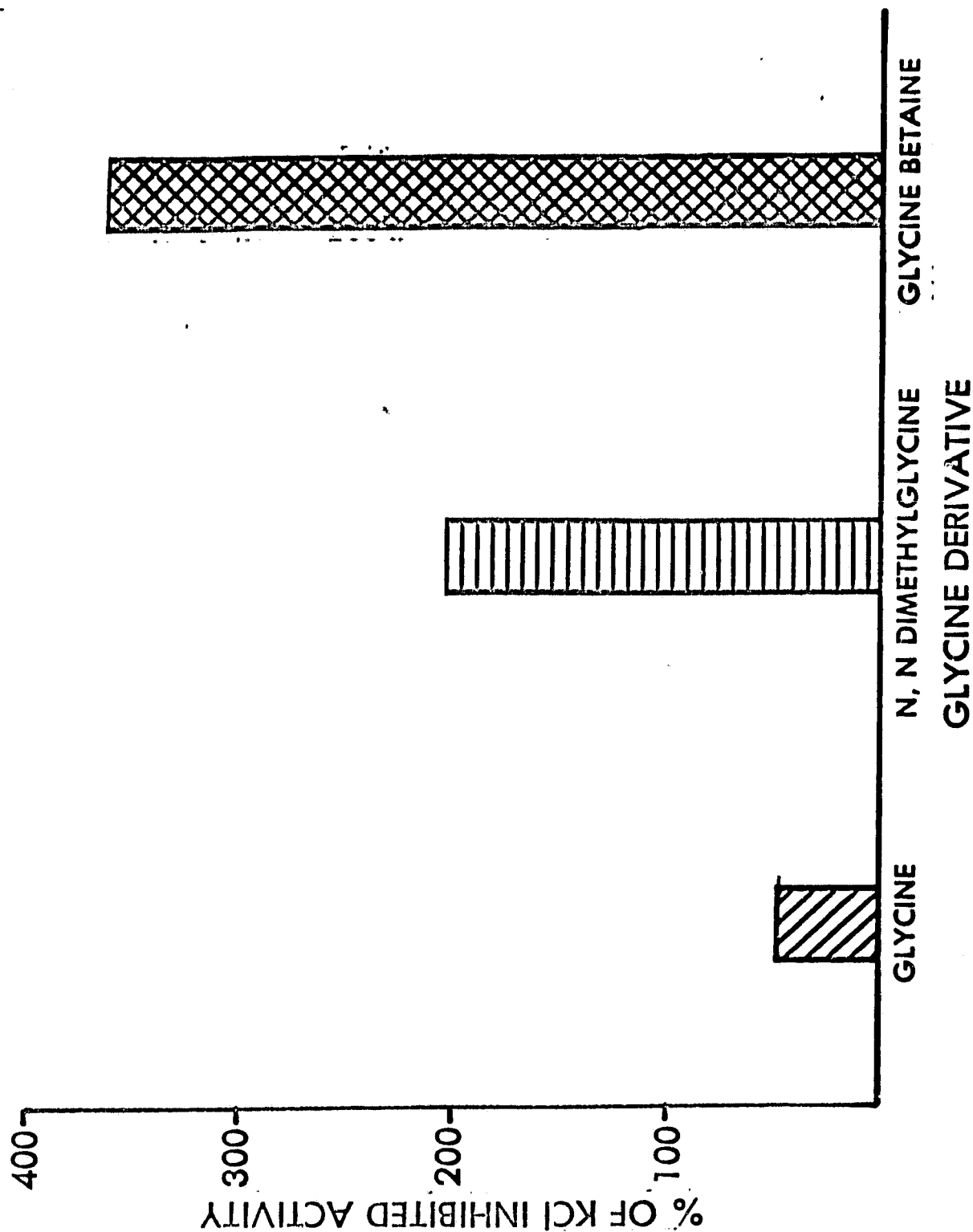


Figure 7. Effect of Glycine Betaine on the Activity of KCl - Inhibited Glucose-6-Phosphate Dehydrogenase from Torula Yeast and the Halotolerant Bacterium, Leuconostoc mesenteroides

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ACTIVITY OF G6PD IN KCl (0.4M FOR *Torula*
AND 1.0M FOR *Leuconostoc*) AS AFFECTED
BY INCREASING GLYCINE-BETAININE

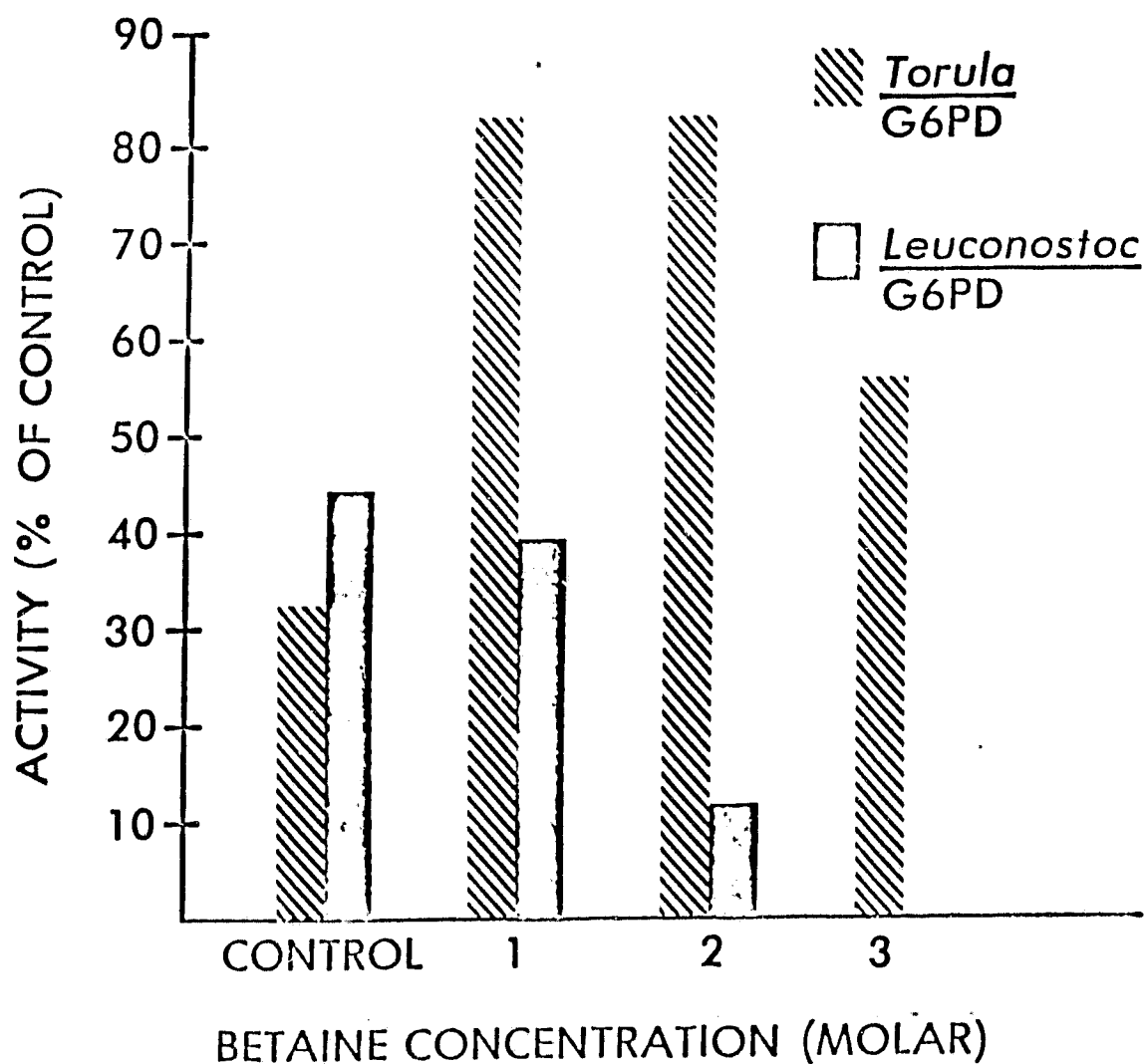


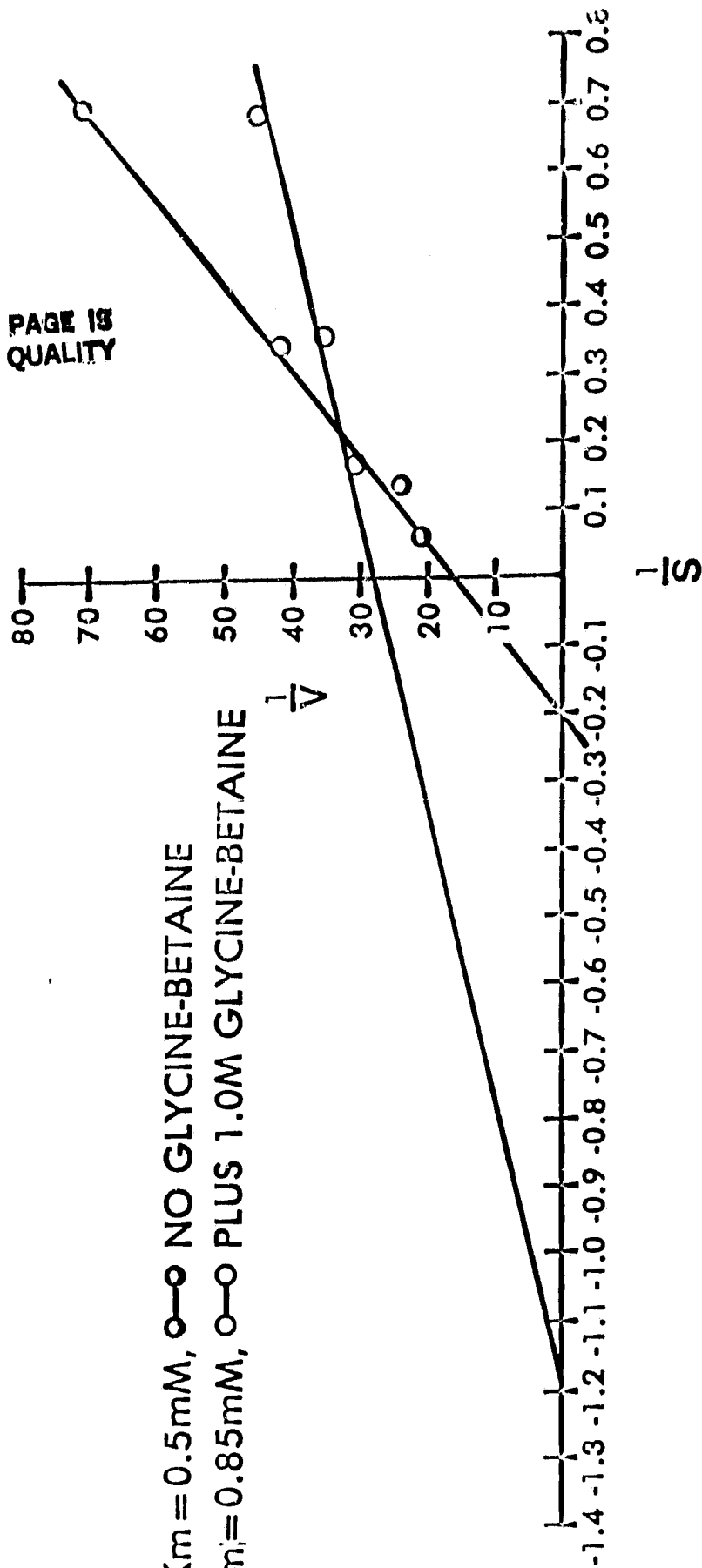
Figure 8. Effect of Glycine Betaine on the Km of Glucose-6-Phosphate Dehydrogenase from Torula and Leuconostoc

GLUCOSE -6-PO₄ DEHYDROGENASE, Leuconostoc mesenteroides

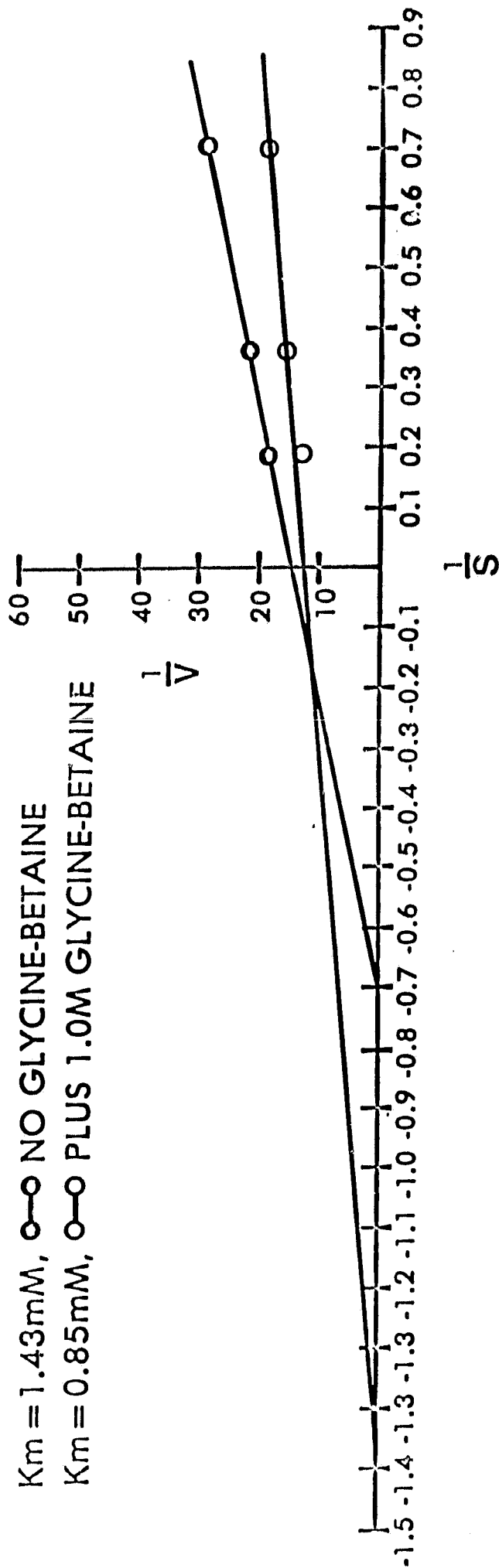
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K_m = 0.5mM, ○—○ NO GLYCINE-BETAINE

K_m_i = 0.85mM, ○—○ PLUS 1.0M GLYCINE-BETAINE



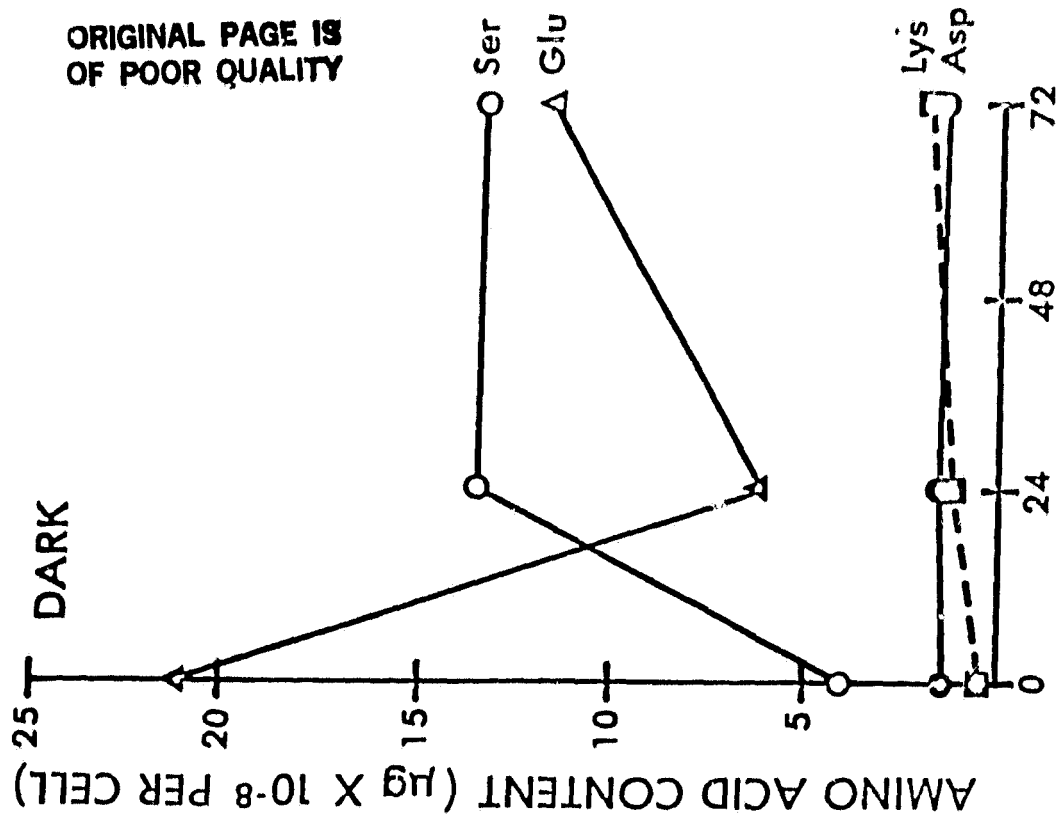
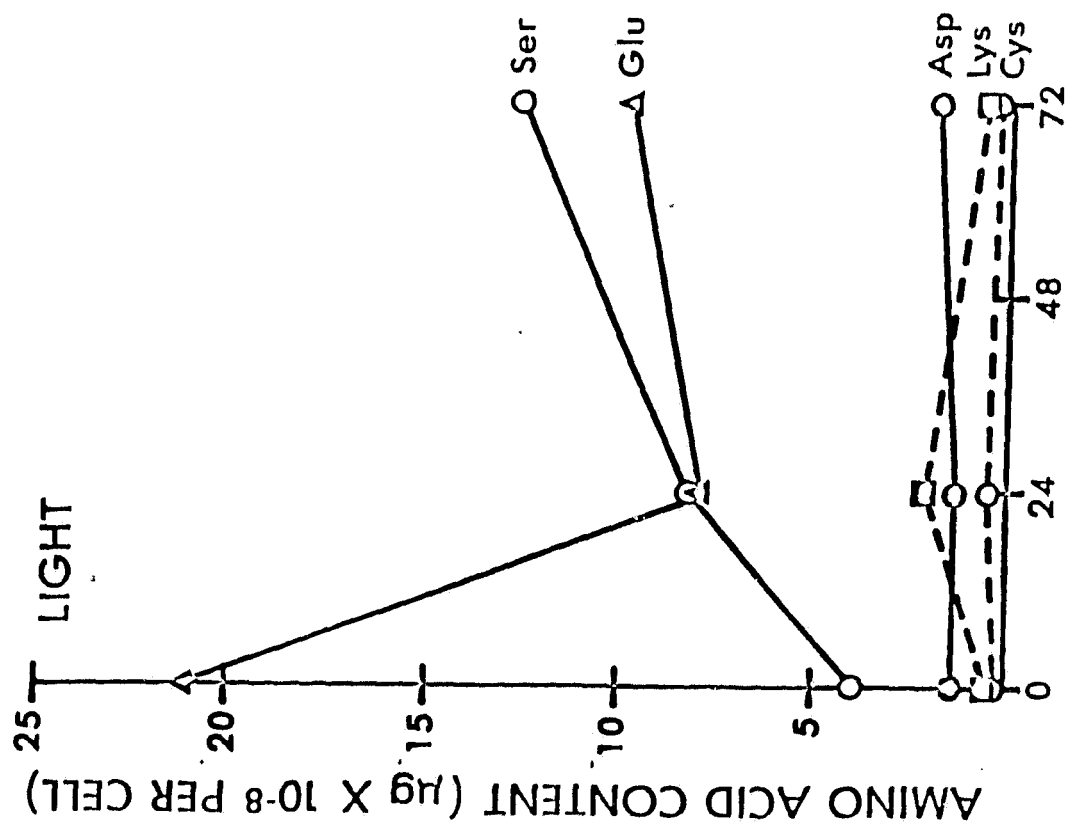
GLUCOSE-6-PO₄ DEHYDROGENASE, Torula YEAST



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Figure 9. Changes in Certain Amino Acids in Aphanothece halophytica Following Upshock in Media Containing Glycylglycine Buffer in the Light and in the Dark

CHANGES IN CERTAIN FREE AMINO ACIDS IN *A. halophytica* FOLLOWING UPSHOCK
 (1.0 M NaCl \rightarrow 2.75 M NaCl - MEDIUM) IN LIGHT AND DARK (GLYCYL - GLYCINE BUFFER)



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Figure 10. Changes in Certain Amino Acids in Aphanothece halophytica
Following Upshock in Media containing MES Buffer in the Light and
in the Dark

CHANGES IN CERTAIN FREE AMINO ACIDS IN A. halophytica FOLLOWING UPSHOCK
 (1.0M NaCl \rightarrow 2.75M NaCl - MEDIUM) IN LIGHT AND DARK (MES BUFFER)

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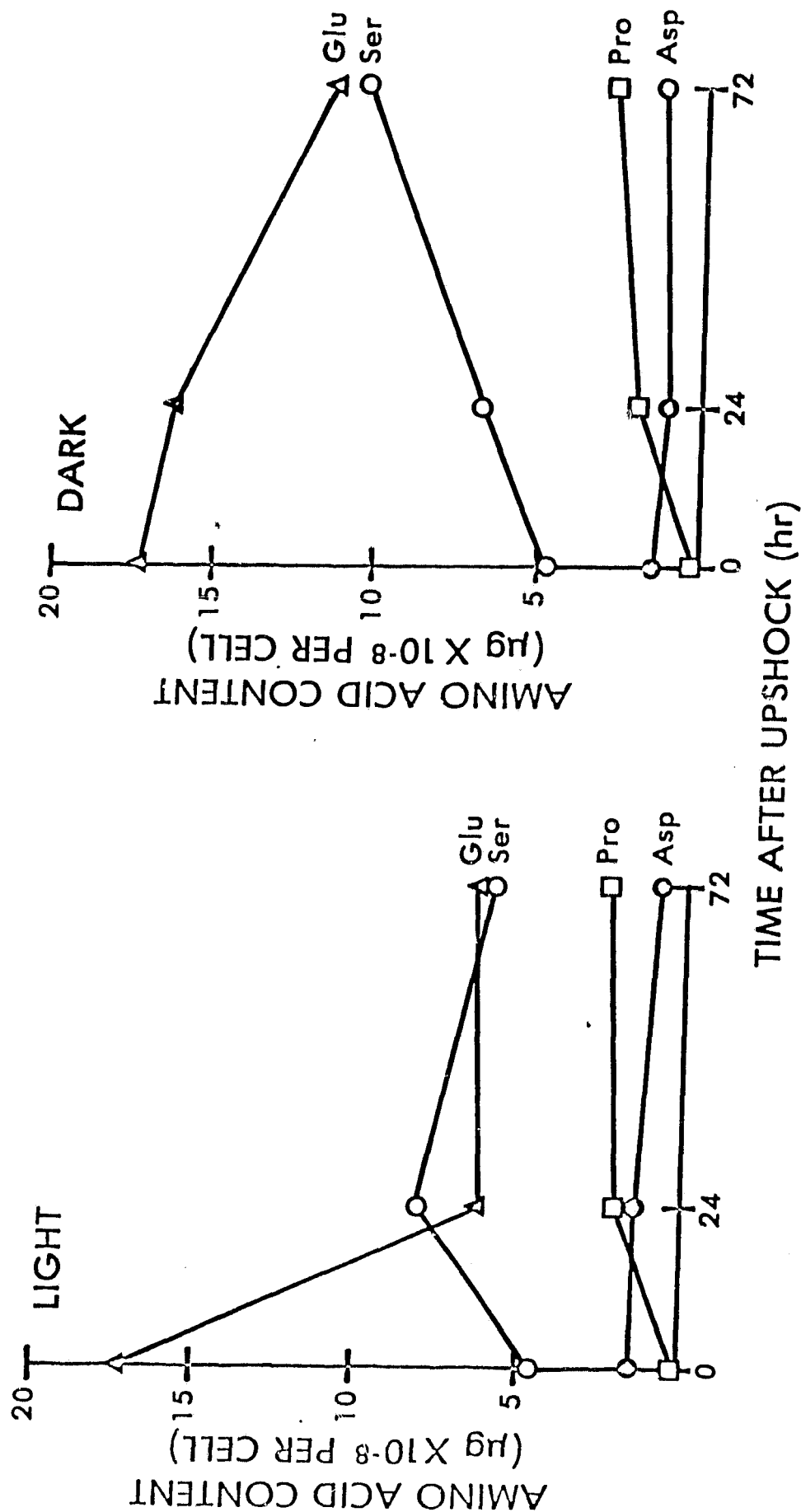


Figure 11. Changes in Certain Amino Acids and Ammonia in Aphanothece
halophytica Following Upshock in Dark, Anaerobic Conditions

CHANGES IN GLUTAMIC ACID, SERINE, ARGININE AND
AMMONIA IN *A. halophytica* FOLLOWING ANAEROBIC
UPSHOCK (1.0M NaCl \rightarrow MEDIUM) IN THE DARK
(GLYCYL-GLYCINE BUFFER)

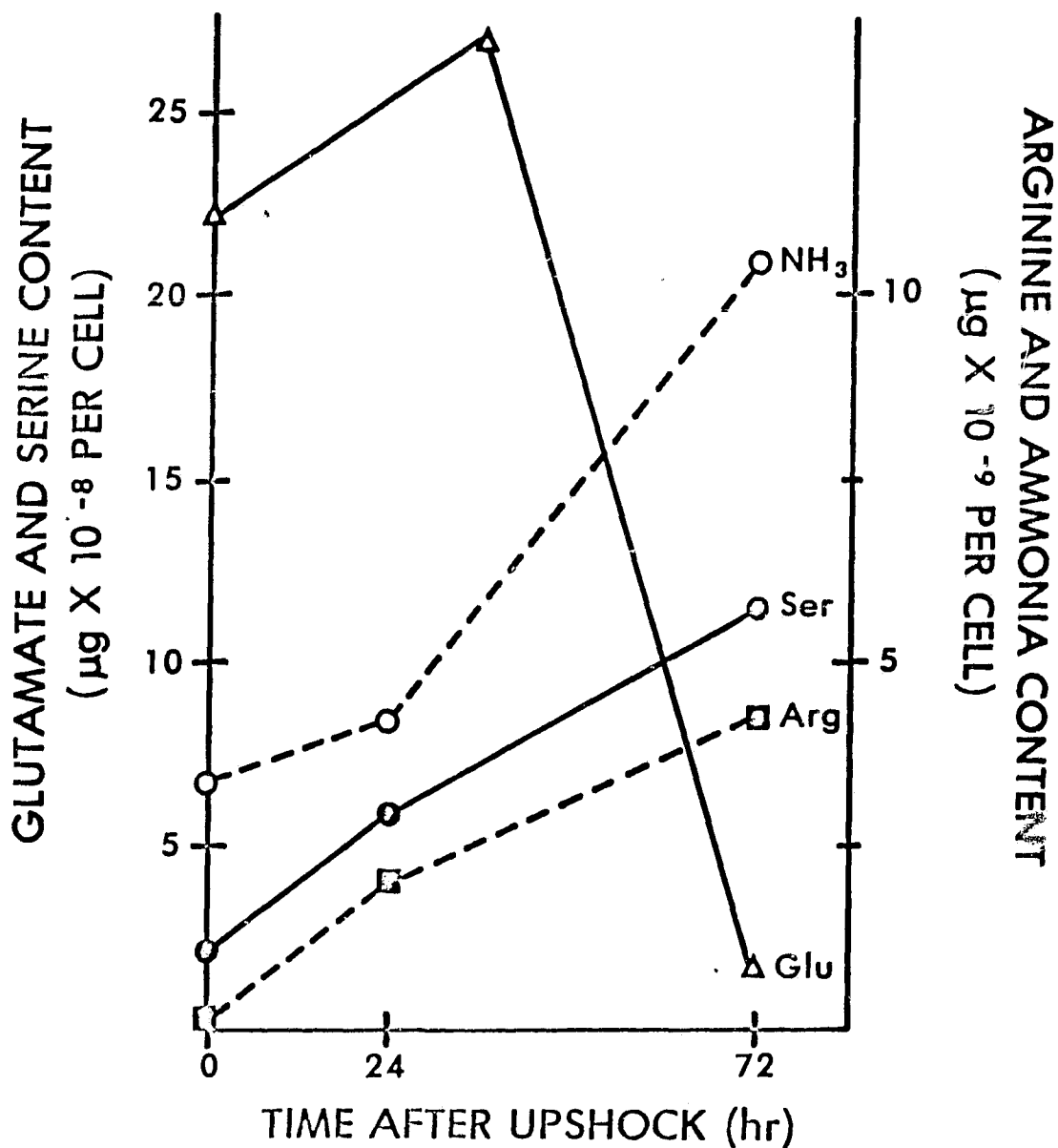


Figure 12. Changes in Total Soluble Carbohydrates in Fischerella Following Upshock in Light and Dark

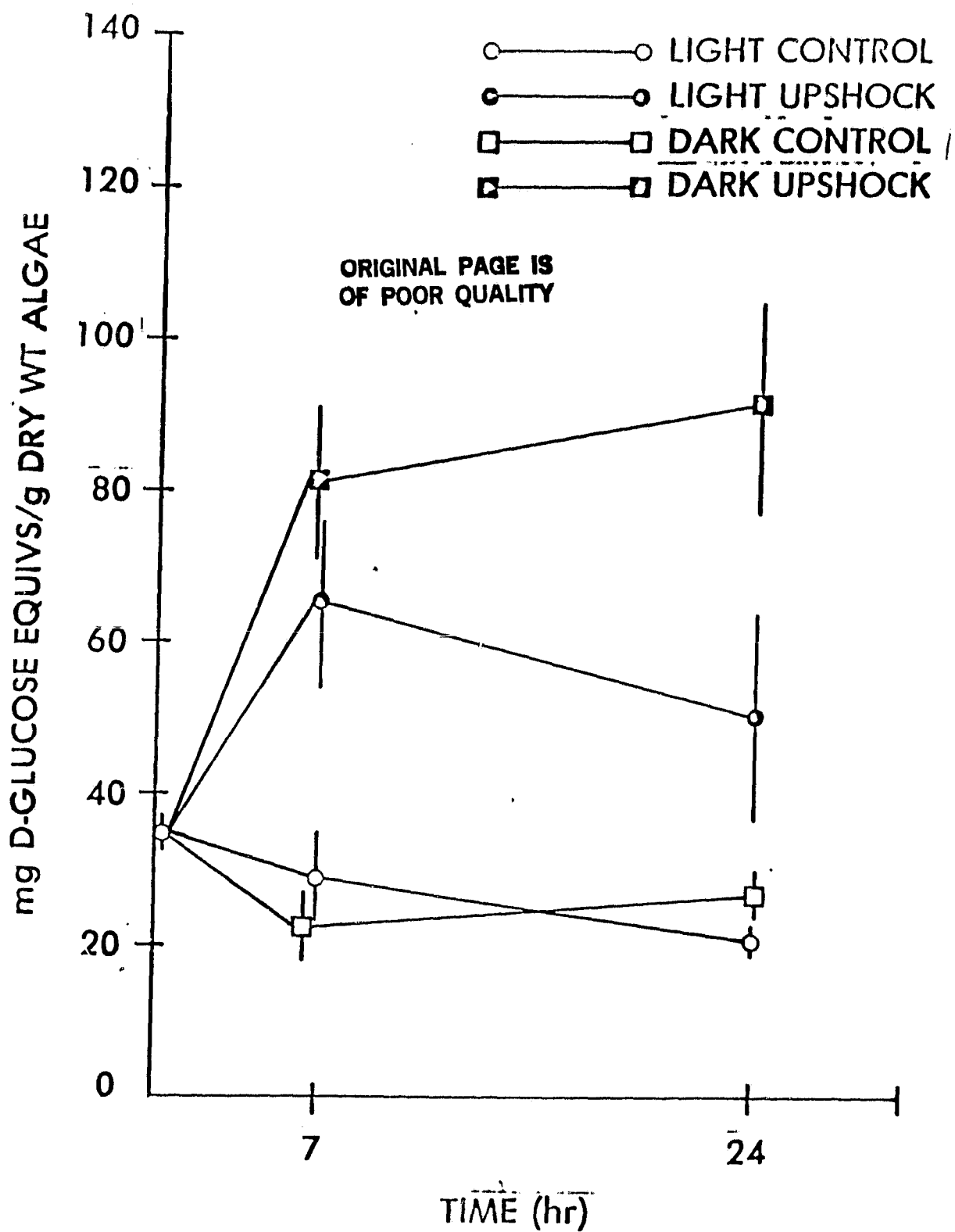


Figure 13. Changes in Reducing Carbohydrates in Fischerella Following Upshock in Light and Dark

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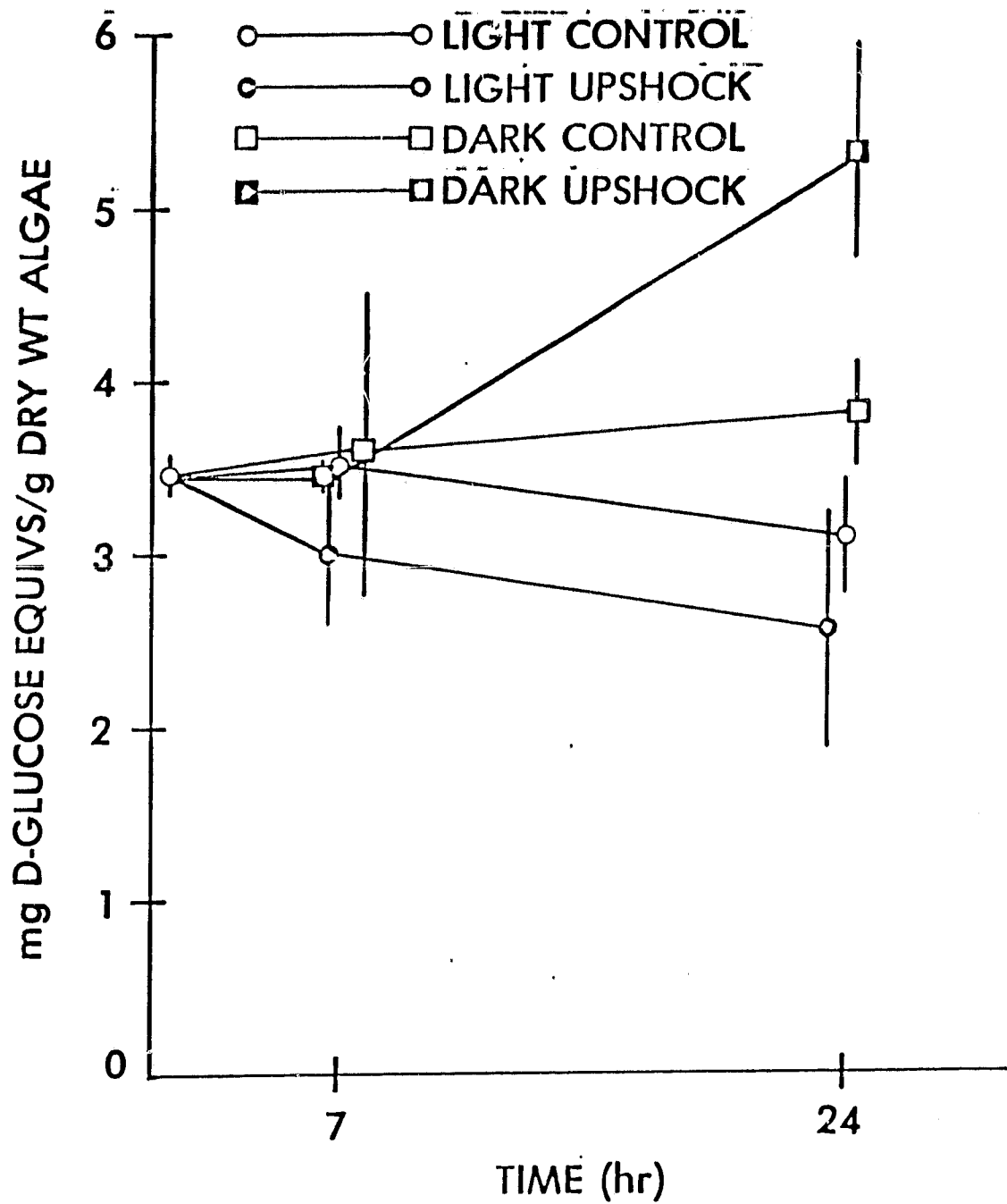


Figure 14. Changes in Total Soluble Carbohydrates in a LPP Cyanobacterium Following Upshock in Light and Dark

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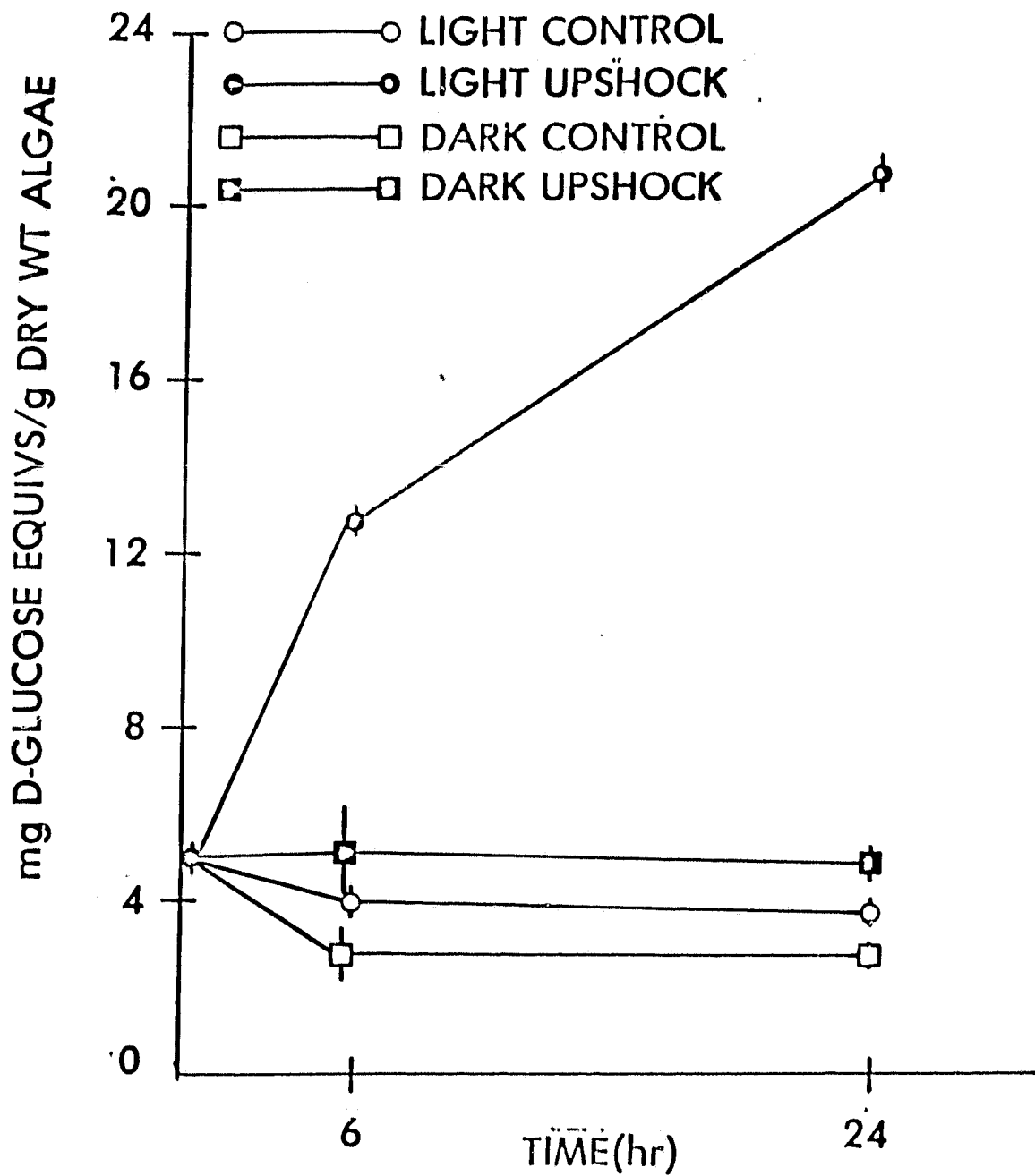


Figure 15. Changes in Reducing Carbohydrates in a LPP Cyanobacterium
Following Upshock in Light and Dark

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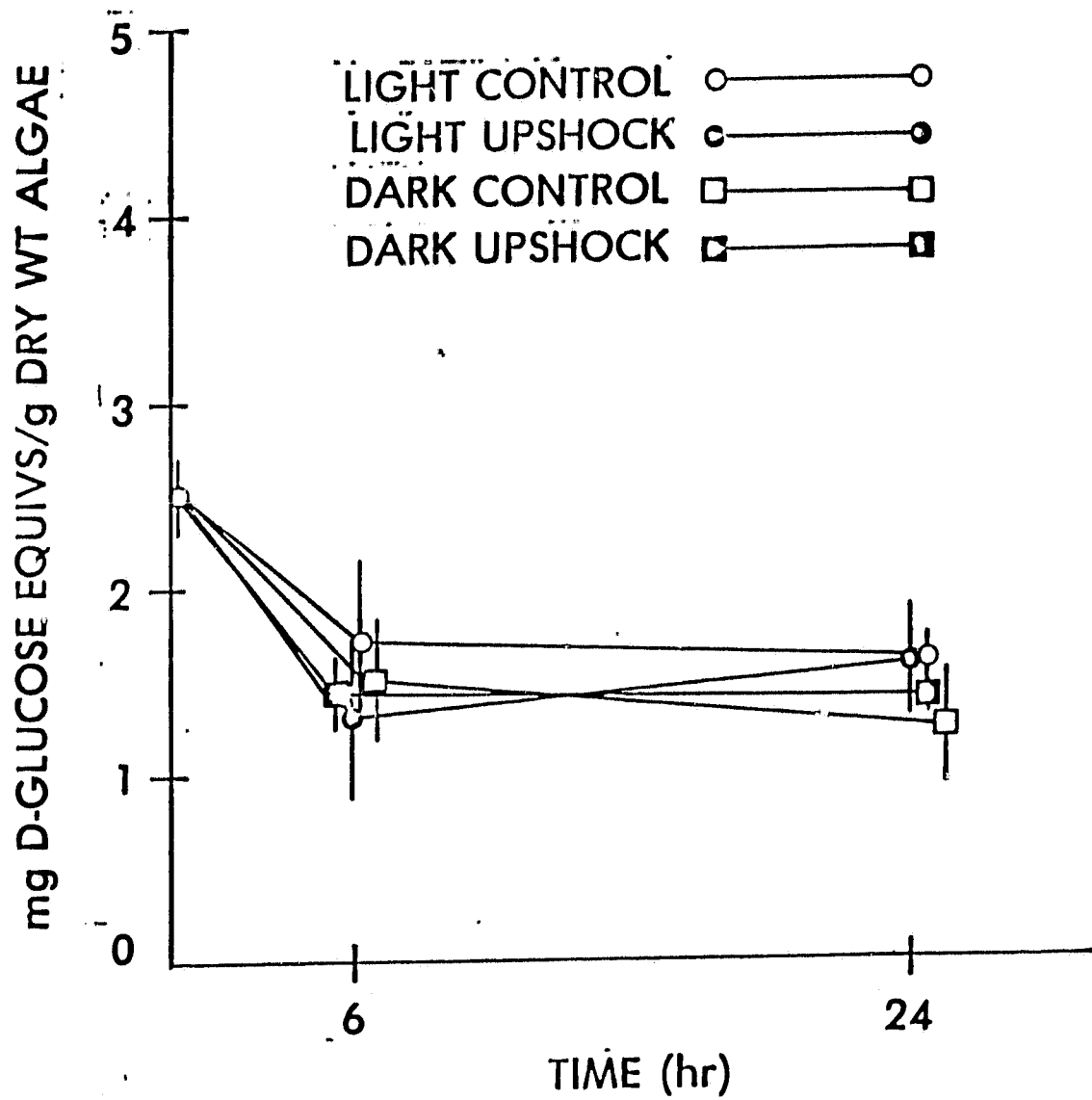


Figure 16. Changes in Total Soluble Carbohydrates in Synechocystis Following Upshock in Light and Dark

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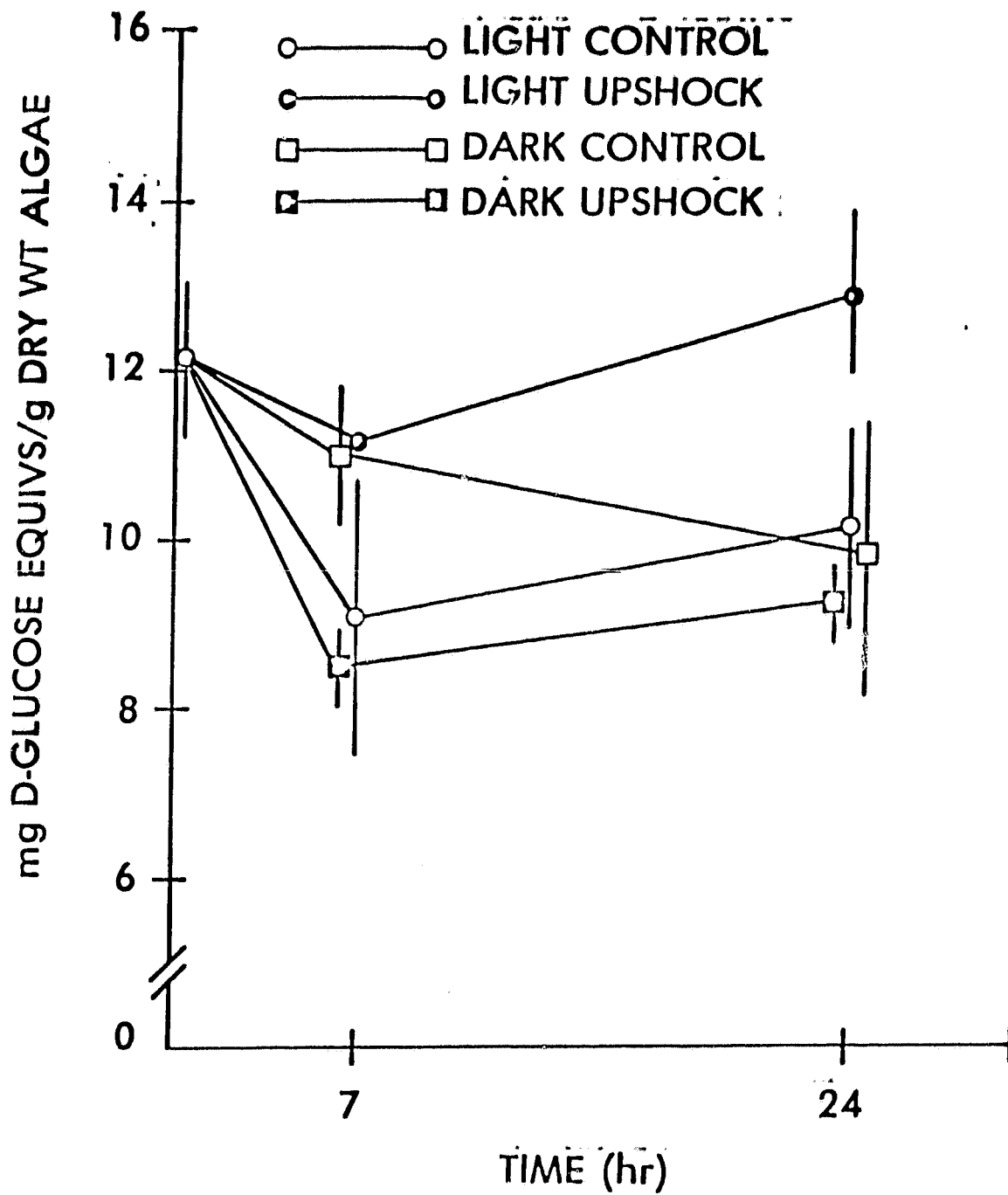


Figure 17. Changes in Reducing Carbohydrates in Synechocystis Following Upshock in Light and Dark

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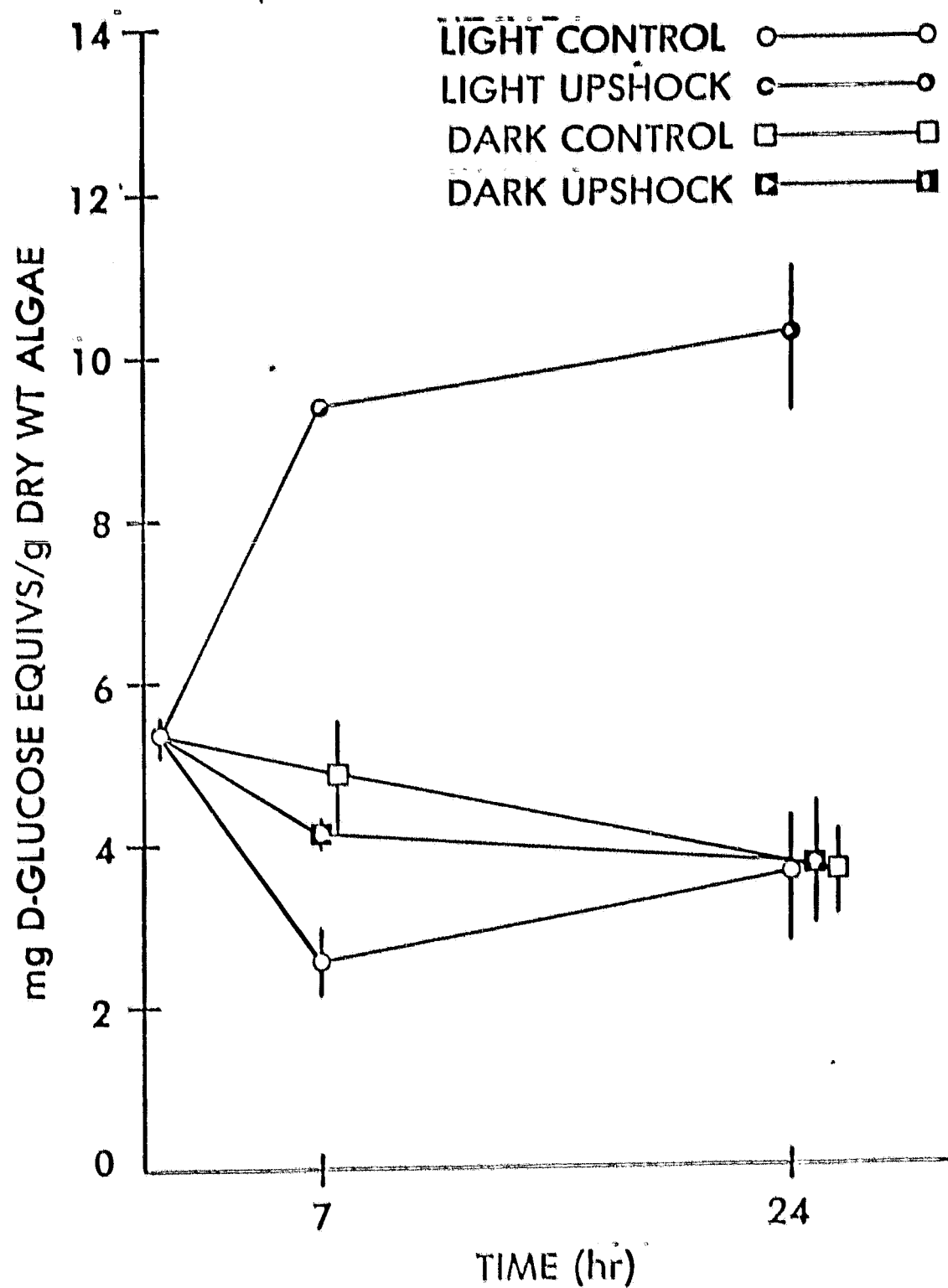


Figure 18. Changes in Total Free Amino Acids in Synechocystis Following Upshock in Light and Dark

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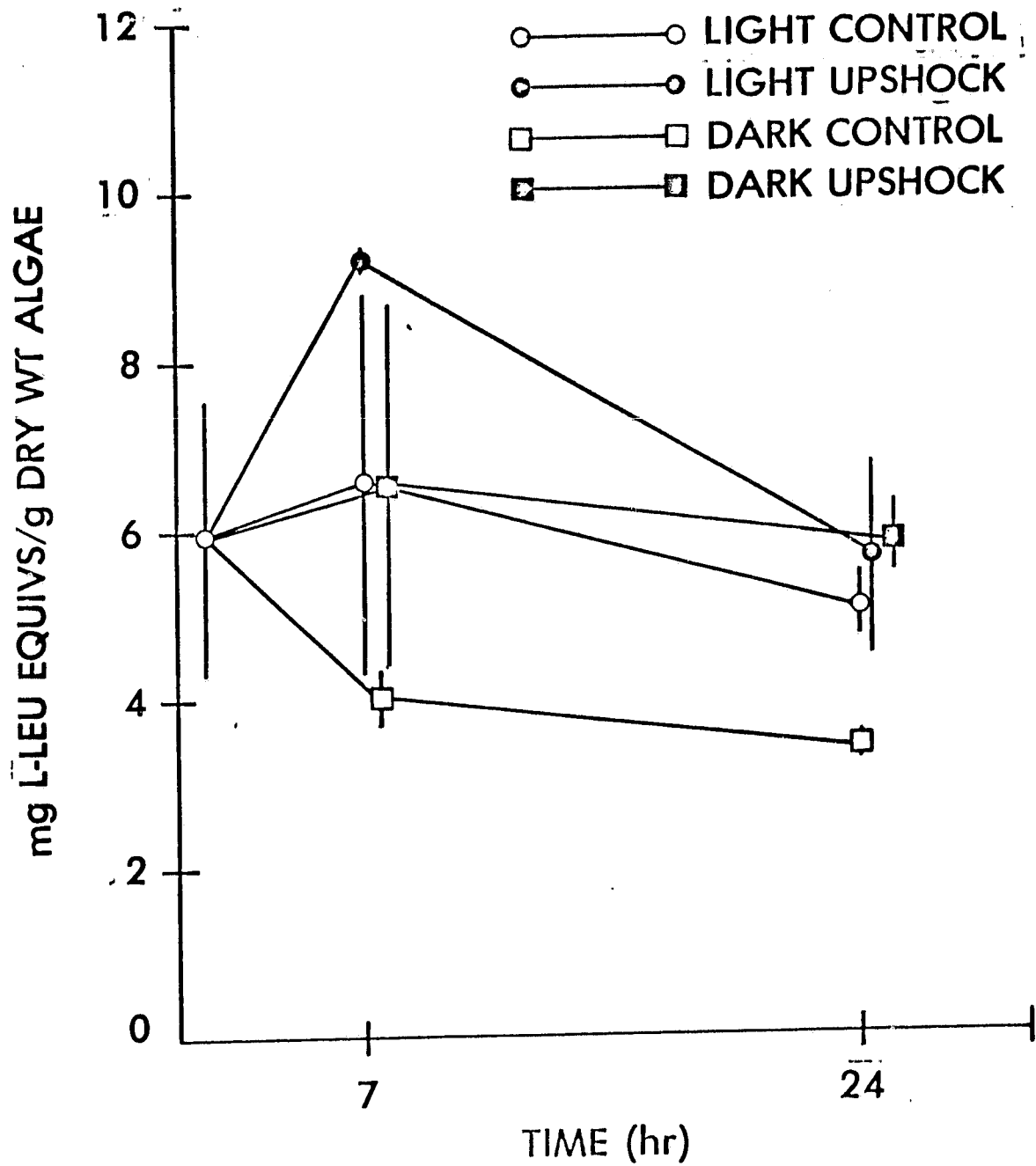


Figure 19. Changes in Total Free Amino Acids in a LPP Cyanobacterium
Following Upshock in Light and Dark

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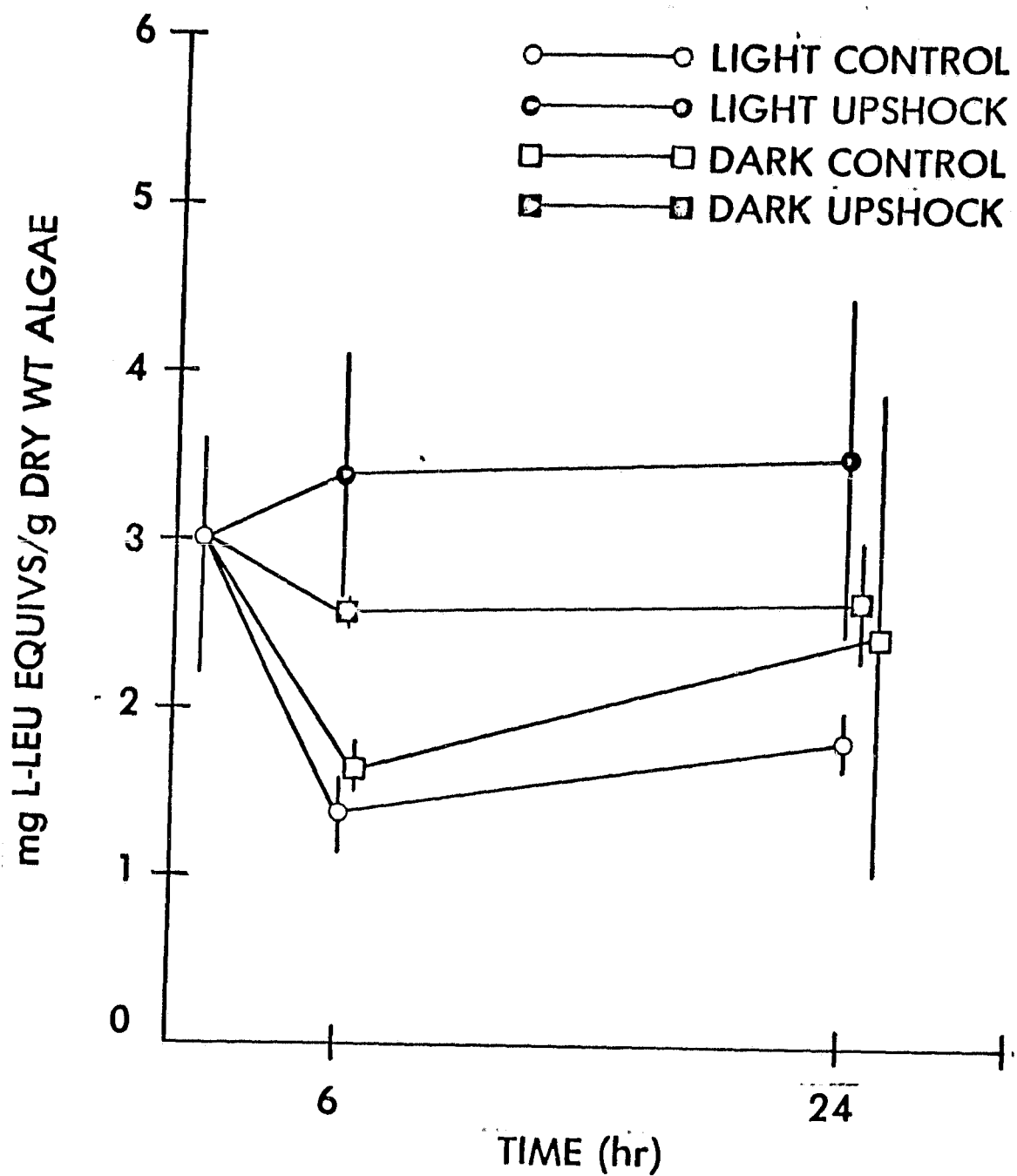


Figure 20. Changes in Total Free Amino Acids in Fischerella Following Upshock
in Light and Dark

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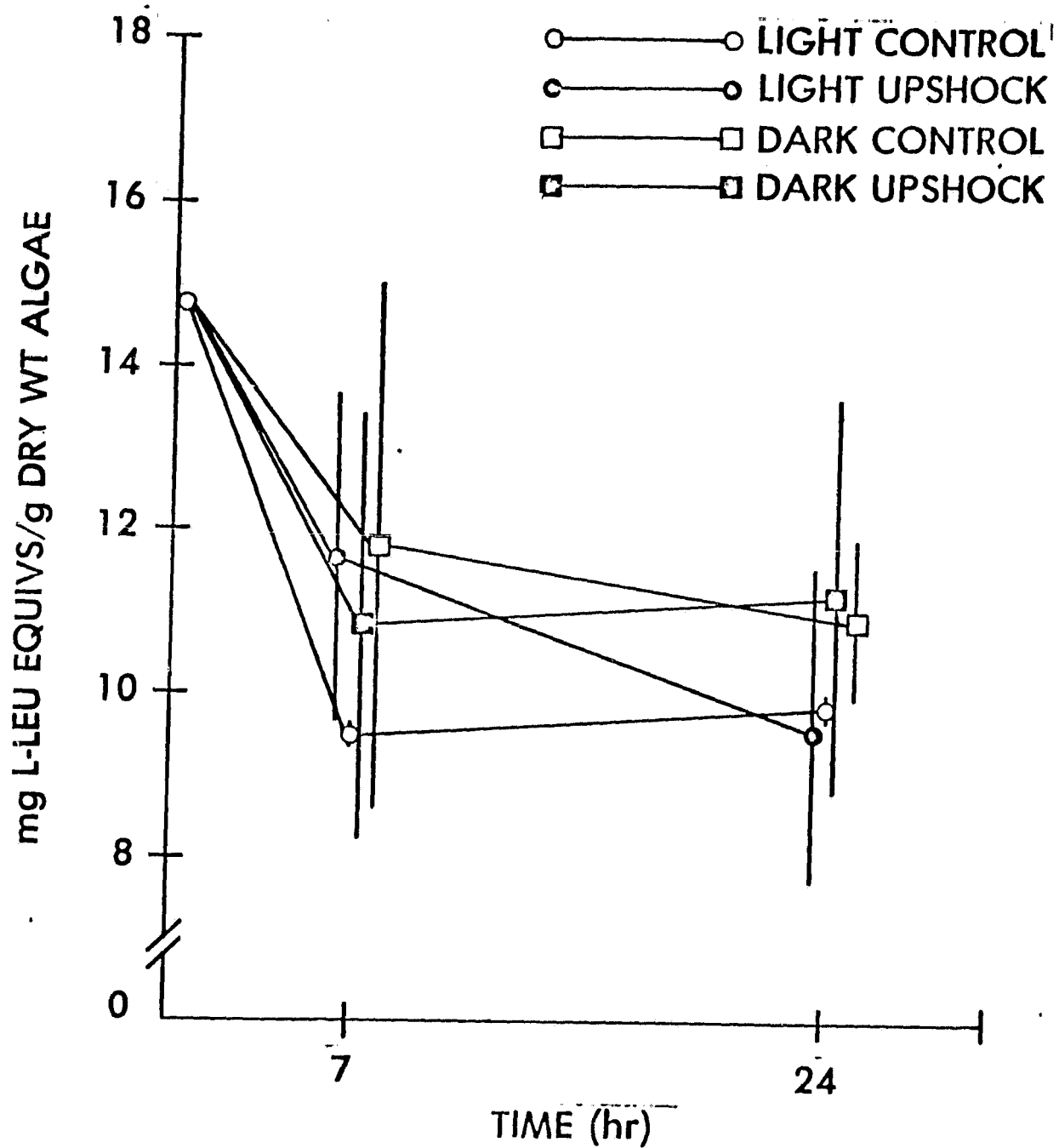
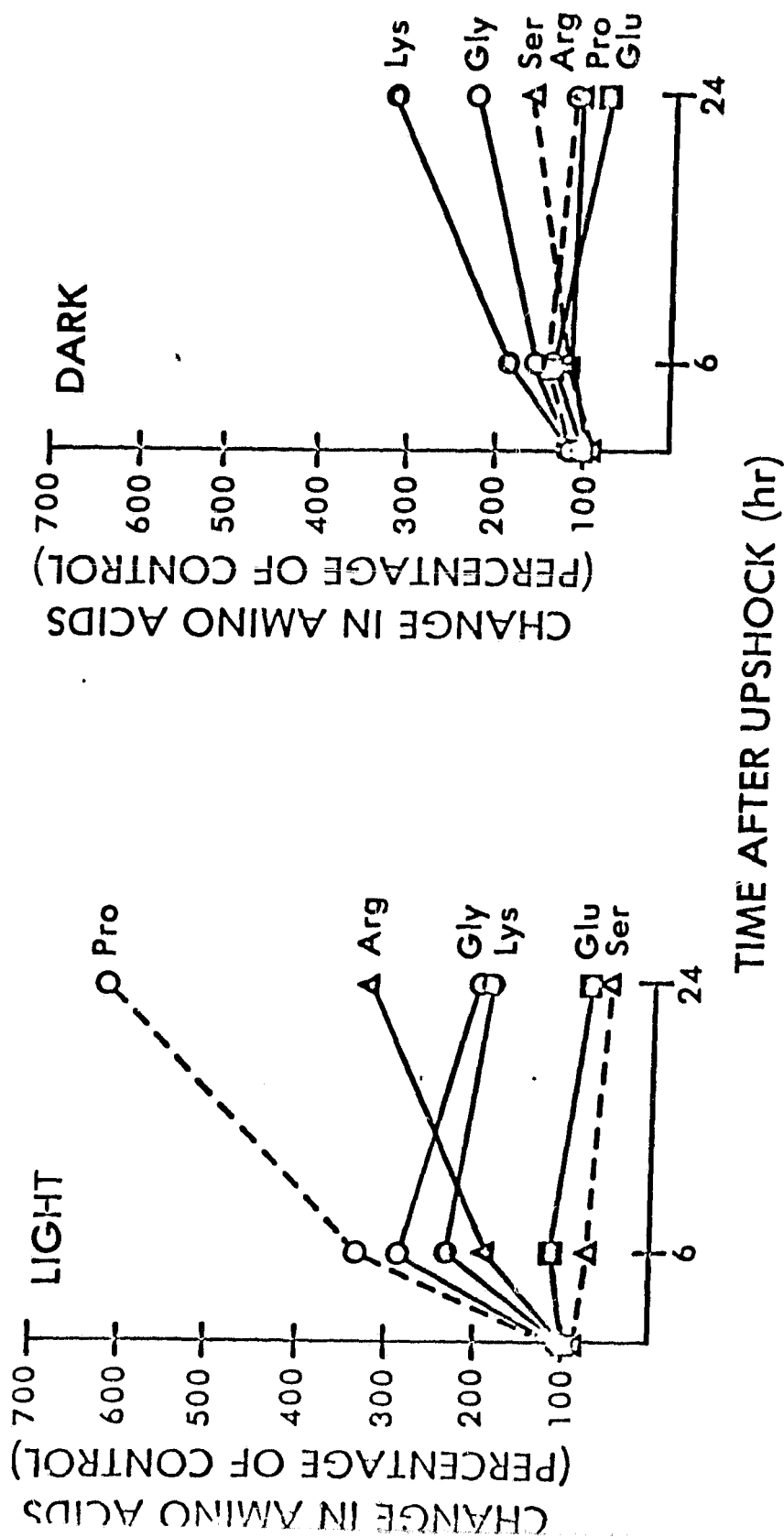


Figure 21. Changes in Certain Free Amino Acids in Synechocystis Following Upshock in Light and Dark

CHANGES IN CERTAIN FREE AMINO ACIDS IN SYNECHOCYSTIS sp. FOLLOWING UPSHOCK IN LIGHT AND DARK



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Figure 22. Changes in Certain Free Amino Acids in a LPP Cyanobacterium
Following Upshock in Light and Dark

CHANGES IN CERTAIN FREE AMINO ACIDS IN LPP *sp.* FOLLOWING UPSHOCK IN LIGHT AND DARK

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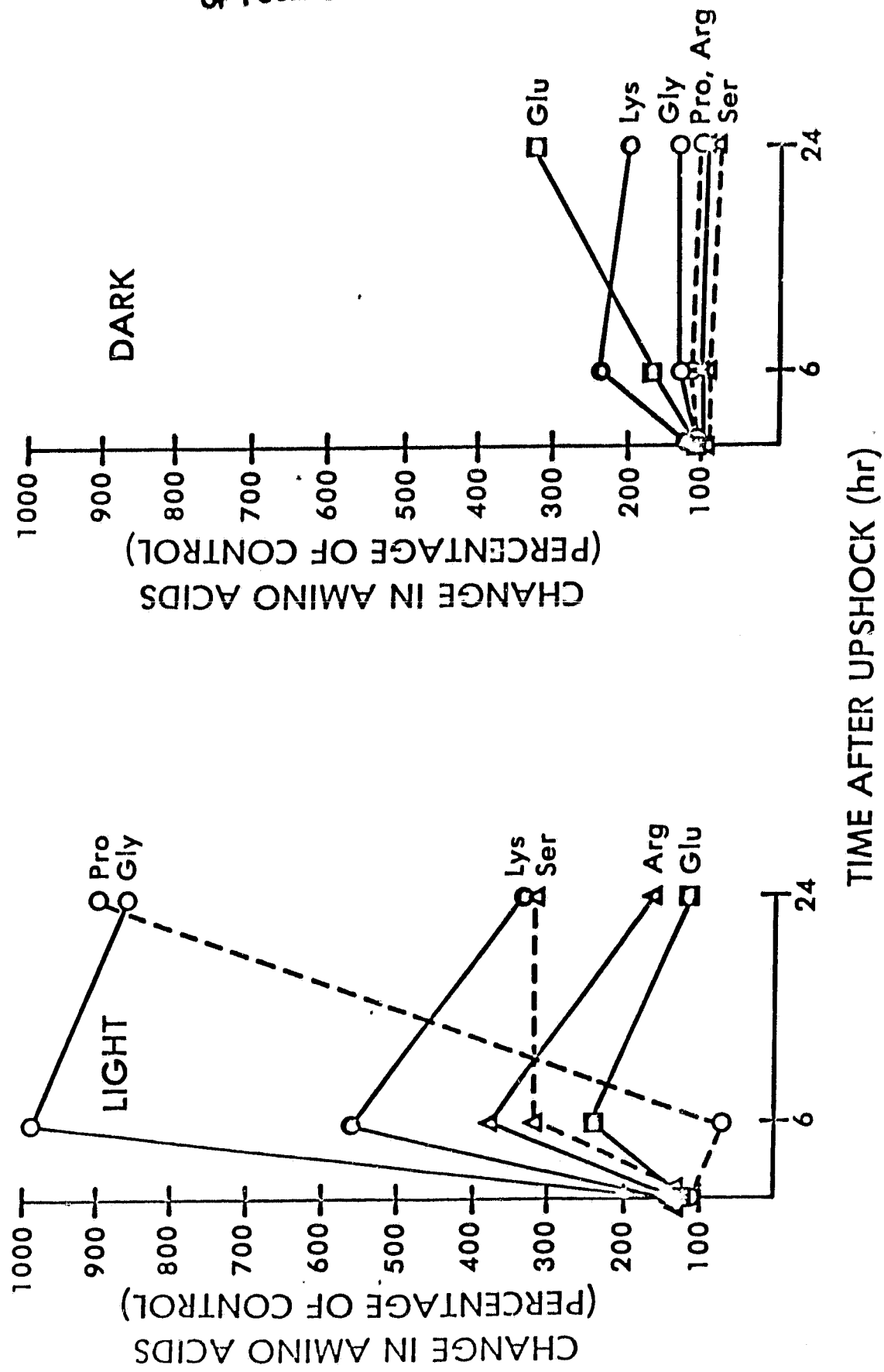
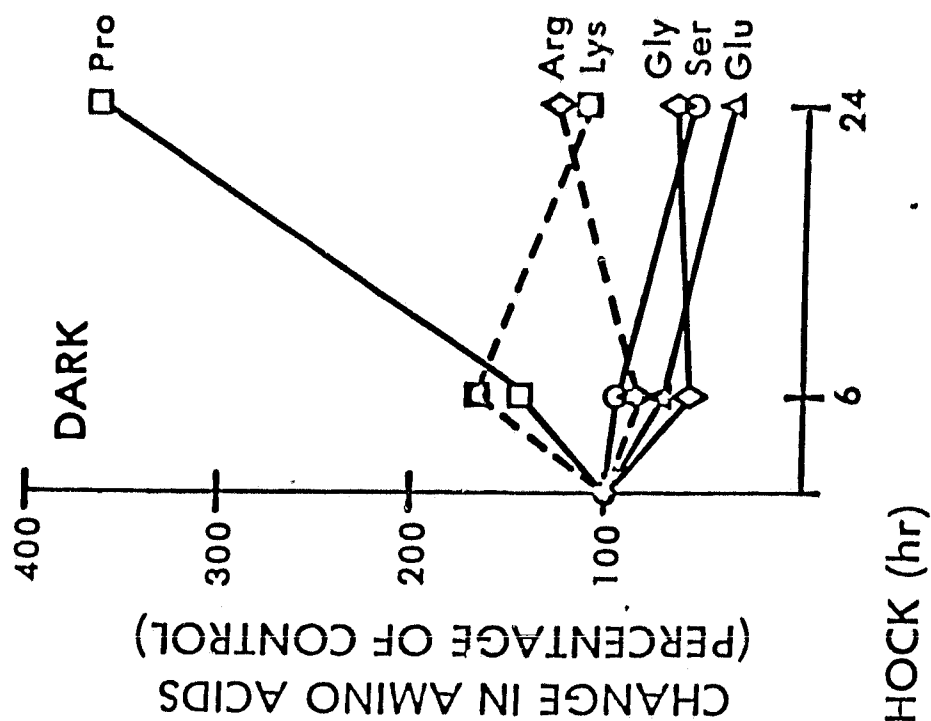
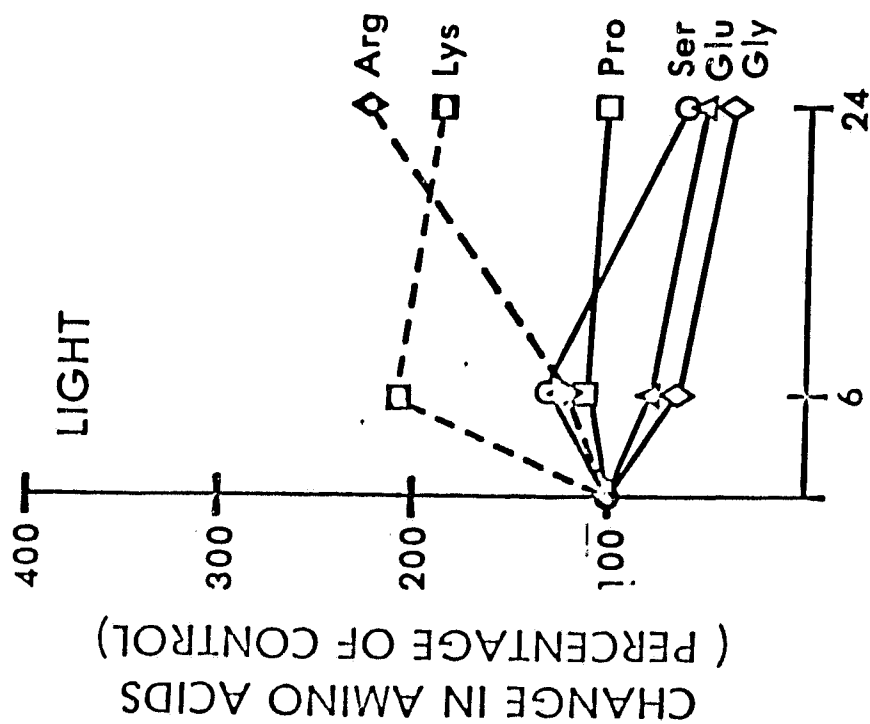


Figure 23. Changes in Certain Free Amino Acids in Fischerella Following Upshock in Light and Dark

CHANGES IN CERTAIN FREE AMINO ACIDS IN FISCHERELLA muscicola FOLLOWING UPSHOCK IN LIGHT AND DARK



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Time course proposed for research designed to achieve the objectives

Year 1:

Culture of all structural and nutritional types of cyanobacteria proposed (see Table 2, original proposal for first year, June 15, 1982 - June 14, 1983); this objective must be met before research on the other objectives progresses);

- (2) Culture of the above strains under conditions that will elicit photoautotrophy, photoheterotrophy, and chemoheterotrophy. This objective will confirm the existence of the appropriate nutritional capabilities of the cyanobacteria, as indicated from the literature.
- (3) Following the changes in osmoregulatory solutes after upshock for approximately one-third of the strains.

Year 1 will then involve partial achievement of Objectives 1 through 3

Year 2 (actually the next eighteen months);

- (1) Following the changes in osmoregulatory solutes after upshock in approximately one half of the organisms,
- (2) Experimentation to achieve goals 5, 6, and 7.

Year 3 (actually the last six months and any proposed extension);

- (1) Following the changes in osmoregulatory solutes after upshock for the last one sixth (approximately) for the cyanobacteria;
- (2) Experimentation designed to achieve goals 8 and 9.

Detailed Breakdown of the Second Period of Funding:

- (1) The first six months of the second eighteen months of funding will involve experimentation to determine the metabolites and metabolic pathways involved in the formation of the osmoregulatory solutes using the C-13 NMR. Concomitant enzymatic studies will be conducted to verify the specific enzymes involved in the formation of these precursor metabolites.
- (2) The subsequent twelve-month period of the second eighteen months of funding will involve experimentation designed to achieve objectives 5, 6, and 7.

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